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MOLECULAR CHARACTERISATION OF THE HERPES SIMPLEX VIRUS 1 LATP2 ENHANCER REGION

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Abstract

HSV1 vectors have previously been produced with the ability to direct long-term expression of a transgene within the nervous system. These viruses have an arrangement of the latency promoter and enhancer regions LAP1 and LATP2 such that LATP2 exerts long-term expression onto LAP1 and an exogenous promoter at the same time, in a back-to-back fashion.

To characterise the LATP2 region, two series of vectors were produced containing deletion mutations of the region placed in different orientations to LAP1 within the context of the original vectors. The vectors were tested *in vitro* and *in vivo* in the PNS and a potentially repressive region within LATP2 was identified. The enhancer activity of the region was also localised to a defined area.

As the HSV1 genome is associated with histones and modification of these is a method of transcriptional control, histone modification could be one mechanism that the virus uses to keep the LAT region active during latency. This was investigated by examining the acetylation of histones associated with the LAT region, including LATP2, at lytic and latent timepoints in an *in vitro* system by ChIP assay. These studies found that although no significant differences in acetylation at different loci of LATP2 was found, the LAT regulatory region generally appears to be more associated with hyperacetylated histones during latency than non-LAT promoters and that this increased acetylation is conferred onto an exogenous promoter when placed within the LAT region.

The findings in this thesis should provide insight into the functioning of the LAT region and may allow the development of improved HSV1 vectors for gene therapy in the nervous system.

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Abbreviations

A	adenosine
BHK	baby hamster kidney
BHV	bovine herpes virus
bp	base pair
BSA	bovine serum albumin
C	cytosine/centigrade
CAT	chloramphenicol acetyltransferase
ChIP	chromatin immunoprecipitation
CMC	carboxymethylcellulose
CMV	cytomegalovirus
CNS	central nervous system
CO_2	carbon dioxide
CPE	cytopathic effect
CPRG	chlorophenolred-ß-D-galactopyranoside
CRE	cyclic AMP response element
DEPC	diethylpyrocarbonate
DMEM	Dulbecco's modified Eagle's medium
DMF	dimethylformamide
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DRG	dorsal root ganglia
ds	double stranded
E	early gene
EBV	Epstein Barr virus
EDTA	ethylenediaminetetra-acetic acid
EGFP	enhanced green fluorescent protein

Egr	early growth
eIF	eukaryotic initiation factor
ER	endoplasmic reticulum
FCS	foetal calf serum
FGM	full growth media
Fig	figure
g	gram
G	guanosine
Gal	galactosidase
gB	glycoprotein B (similarly for other glycoproteins)
GFP	green fluorescent protein
GDNF	glial-derived neurotrophic factor
H	histone
Hr(s)	hour(s)
HAT	histone acetyltransferase
HBSS	Hank's balanced salt solution
HCF	host cell factor
HDAC	histone deacetylase
HEBES	HEBES transfection buffer
HMBA	hexamethylene bisacetamide
HMG	high motility group
HMT	histone methyltransferase
HSDNA	herring sperm DNA
HSV	herpes simplex virus
HVEM	herpesvirus entry mediator
IAA	isoamyl alcohol
ICP	infected cell polypeptide
IE	immediate early gene

IFN	interferon
Ig	immunoglobulin
IP	immunoprecipitation/immunoprecipitated
IRES	internal ribosomal entry site
K	lysine
Kb	kilobase
KDa	kilodalton
L	late gene
LAP	Latency active promoter
LAT	latency associated transcript
LATP	LAT promoter
LB	Luria Bertani
LMP	low melting point
LTE	long-term expression
LTR	long-terminal repeat
M	molar
mg	milligram
MHC	major histocompatibility complex
min(s)	minute(s)
mL	millilitre
mm	millimetre
mM	millimolar
MMLV	murine moloney leukaemia virus
MOI	multiplicity of infection
mRNA	messenger RNA
MS	master stock
MW	molecular weight
NaCl	sodium chloride

NaOH	sodium hydroxide
ND7	mouse neuroblastoma DRG fusion cell line
ng	nanogram
NGF	nerve growth factor
nM	nanomole
NP40	nonidet P40
nt	nucleotide
oct	octamer
OD	optical density
O/N	overnight
ORF	open reading frame
ori	origin of replication
p	plasmid
pA	polyA; polyadenylation
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
pfu	plaque forming units
p.i.	post-infection/post-injection
PML	pro-myeolytic leukaemia
PNS	peripheral nervous system
pol	polymerase
qPCR	quantitative PCR
R	arginine
RL	repeated long
RNA	ribonucleic acid
rpm	revolutions per minute
RS	repeated short

RSV	rous sarcoma virus
RT	room temperature
S	serine
SD	standard deviation
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
S/N	supernatant
SSC	standard sodium citrate
SV40	simian virus 40
T	thymidine
TAE	tris-acetate EDTA buffer
TAP	transporter of antigen processing
TE	Tris/EDTA
TF	transcription factor
TG	trigeminal ganglia
TIF	trans-inducing factor
TK	thymidine kinase
T_m	melting temperature
TNF	tumour necrosis factor
Tris	tris-acetate EDTA buffer
TSA	trichostatin A
U_L	unique long
U_S	unique short
USF	upstream stimulatory factor
V	volt
vhs	virion host shut-off
VP	virion protein
v/v	volume for volume

VZV	varicella zoster virus
WT	wild type
w/v	weight for volume
X-Gal	4-chloro, 5-bromo, 3-indolyl-β-galactosidase
μg	microgram
μL	microlitre
μM	micromole
-ve	negative
+ve	positive

CHAPTER 1:

INTRODUCTION

1.1 HERPES SIMPLEX VIRUSES

The herpesviruses are classified into 3 types: α, β and γ. This distinction is made by the tissue tropism, cytopathology and the duration of the replicative cycle of the virus.

The α-herpesviruses infect humans, have a short replicative cycle and are neurotropic, although *in vivo* and *in vitro* they have been shown to infect a broad range of cell types as well as neurons. Members of the α subfamily are herpes simplex virus 1 (HSV1), herpes simplex virus 2 (HSV2) and varicellar zoster virus (VZV).

The β- and γ-herpesviruses have a slower replicative cycle and infect a narrower range of cells, primarily those of lymphatic or glandular origin. β-herpesviruses are typified by cytomegalovirus (CMV) and γ-herpesviruses by epstein barr virus (EBV).

All herpesviruses are able to establish and maintain latent infections for the lifetime of the host, with the ability to reactivate in response to appropriate stimuli and thus cause symptoms long after the time of primary infection.

1.1.1 HSV 1 and 2

HSV1 is endemic within the human population, with approximately 80% of individuals testing positive for antibodies to HSV1, although a considerably lower percentage show symptoms of the disease. HSV2 is a little less prevalent within the population (Rouse and Gierynska 2001). Both viruses infect mucosal surfaces of either the orofacial (HSV1) or genital (HSV2) region and produce the symptoms of cold sores or genital herpes respectively (Whitley 1996). Very rarely they can cause ocular infection or infect the central nervous system (CNS) and cause encephalitis (Kennedy 1984).

1.1.2 HSV1 Biology

HSV1 is a large (150-200nm in diameter) enveloped virus with a distinct virion structure characteristic of the herpesviruses. An HSV virion consists of a core containing viral DNA, an icosahedral capsid; a tegument containing viral proteins (about 65% of the virion mass consisting of approximately 14 proteins) and a lipid bilayer envelope through which protrude viral glycoprotein spikes (Wildy *et al.* 1960). The virion structure is illustrated in figure 1-1a.

The viral genome is a linear double-stranded DNA molecule packaged within the capsid (Becker *et al.* 1968). It is approximately 152Kb long and encodes at least 80 genes either essential for replication or non-essential (*i.e.* they can be deleted without affecting virus growth). The genome is composed of long and short unique segments (U_L and U_S respectively) flanked by terminal repeat regions (R_L and R_S respectively). The unique segments are covalently linked and can invert relative to each other to generate four possible isoforms that are equally functional and exist in equimolar concentrations in infected cells (Hayward *et al.* 1975; Jacob *et al.* 1979; Roizman 1979).

HSV1 contains 3 origins of replication: one in the U_L region (called OriL) and one in each of the R_S regions (OriS). Either Ori is sufficient for replication (Igarashi *et al.* 1993; Polvino-Bodnar *et al.* 1987). The structure of one viral genome isoform is shown in figure 1-1b.

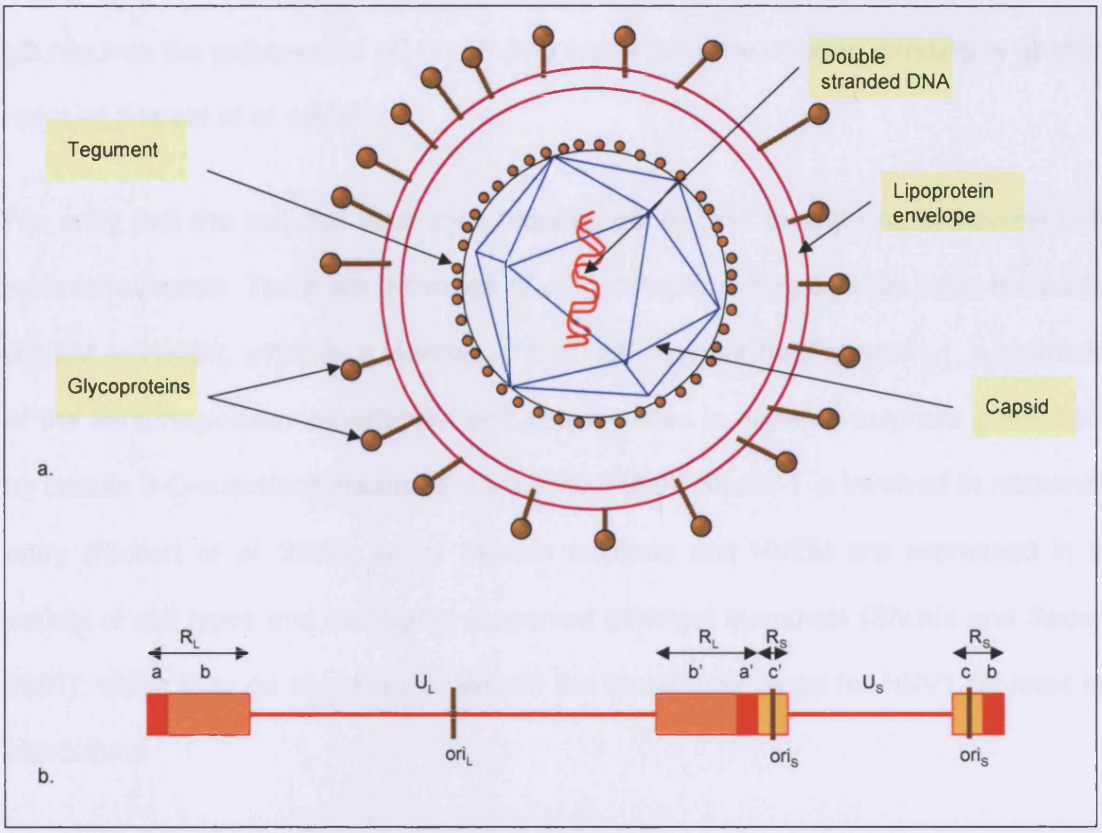


Figure 1-1 Diagram of the HSV1 virion and the structure of the genome

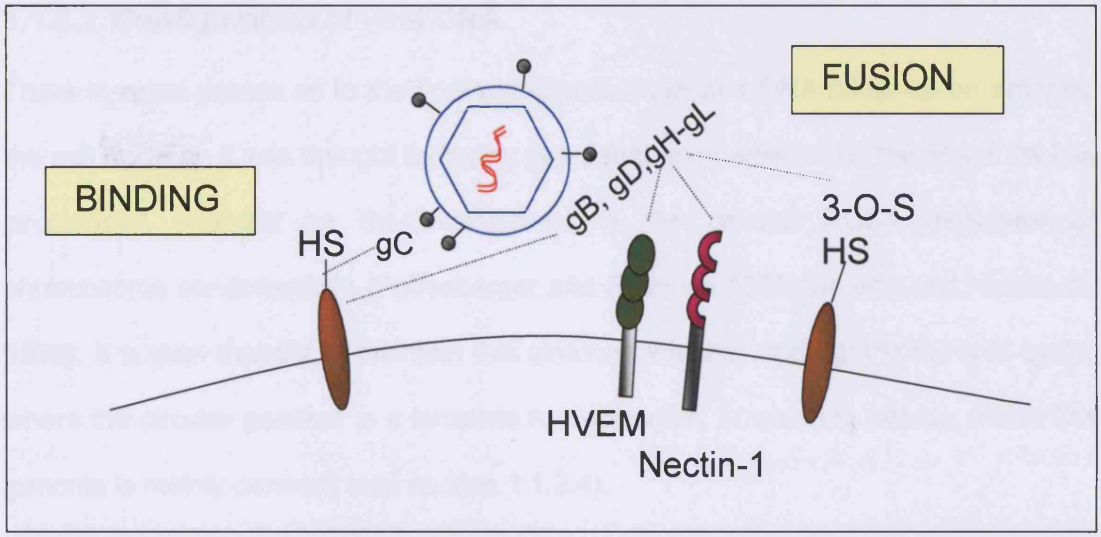
a. The HSV virion consists of double-stranded DNA protected by a nucleocapsid and surrounded by the tegument proteins. The lipoprotein envelope studded with glycoproteins surrounds the internal structures.
b. The HSV genome is organised into the unique long and short regions flanked by the long and short repeats (a, b and c). The 'a' regions contain sequences for packaging. The three origins of replication are also shown.

1.1.2.1 Cell attachment and entry

HSV1 infects cells of the mucosal epithelia, replicates and following which the virus enters nearby nerve endings. Initial contact of HSV with a cell is with heparan sulphate (HS) of the cell surface proteoglycan, to which the virus binds (Shukla and Spear 2001). Two of the virion glycoproteins, gC or gB are able to bind to heparan sulphate. gB requires the presence of gC for binding and if both are missing, binding is greatly reduced (Herold *et al.* 1994).

For entry into the cell, the virus then requires gD to bind to any one of several cell surface receptors. There are 3 classes of entry receptors: herpes virus entry mediator (HVEM or HVEa), which is a member of the TNF receptor family; nectin-1, a member of the immunoglobulin superfamily; and specific sites in heparan sulphate generated by certain 3-O-sulfotransferases (Shukla *et al.* 1999). Nectin-1 is involved in neuronal entry (Richart *et al.* 2003), whilst heparin sulphate and HVEM are expressed in a variety of cell types and are highly conserved amongst mammals (Shukla and Spear 2001), which may go someway to explain the broad host range for HSV1, at least in cell culture.

The binding of gD to one of the cell-surface receptors triggers fusion of the viral envelope with the cell membrane (Morgan *et al.* 1968). This requires the action of the glycoproteins gB and a heterodimer of gH-gL, although the exact mechanism by which fusion occurs is unknown. The interaction of viral glycoproteins and cell-surface receptors is illustrated in figure 1-2.



Adapted from (Spear 2004)

Figure 1-2 Cell surface receptors and viral glycoproteins that participate in HSV1 entry

Five of the viral glycoproteins are involved in entry of the host cell. Binding is mediated by gB and/or gC to heparan sulphate (HS) chains, which facilitates gD binding with one of the cell surface receptors, which in turn leads to fusion.

Following fusion, the viral nucleocapsid and tegument enter the cell cytoplasm. The nucleocapsid and some tegument proteins (e.g. VP16) are transported to the nucleus via microtubules by the cellular motor protein dynein (Sodeik *et al.* 1997). Other tegument proteins such as vhs and US11 remain in the cytoplasm. Once at the nucleus, the nucleocapsid empties the DNA into the nucleus through a nuclear pore demonstrated by the fact that empty viral capsids have been shown to accumulate at the nuclear envelope and associate with nuclear pore structures (Batterson *et al.* 1983; Sodeik *et al.* 1997).

Once in the nuclei, the viral genome may participate either in a lytic or a latent infection.

1.1.2.2 Configuration of viral DNA

There is some debate as to the conformation that the viral DNA takes up on entering the cell nucleus. It was thought for many years that soon after entry, the linear DNA is circularised, possibly by the involvement of the cellular RCC1 (regulator of chromosome condensation) (Poffenberger and Roizman 1985; Umene and Nishimoto 1996). It is then thought to maintain this structure whether undergoing the lytic cycle, where the circular genome is a template for replication, or entering latency where the genome is mainly dormant (see section 1.1.2.4).

However some evidence has recently been provided for a linear template for viral DNA replication (Jackson and DeLuca 2003) regulated by ICP0, as in one study, circular genomes were only seen with ICP0 mutants where lytic replication is inhibited. This data however has recently been challenged by experiments that again showed circular genomes early in infection that are not inhibited by ICP0 (Strang and Stow 2005).

Whichever conformation the viral DNA takes, it is deposited at ND10 or PML bodies within the host nucleus where IE gene transcription ensues (Maul and Everett 1994). It is not known whether the genomes diffuse to the ND10 sites or are deposited there by the cell, but this occurs in the absence of prior viral protein synthesis.

It was previously thought that the viral genome was not associated with nucleosomes during lytic replication (Leinbach and Summers 1980). However, recent experiments repeating this work showed that the viral DNA does indeed take on a nucleosomal structure early in infection and also that it has associated histone modifications consistent with an active genome (Kent *et al.* 2004). The role of histone modifications in the regulation of the HSV genome will be discussed in chapter 5.

1.1.2.3 The lytic cycle

During lytic infection, viral proteins in the tegument direct cellular resources away from host cell metabolism to the virus' advantage. E.g. vhs (UL41) shuts off cellular protein synthesis and degrades mRNAs (Fenwick and McMenamin 1984; Schek and Bachenheimer 1985; Strom and Frenkel 1987), although this includes viral mRNAs as well as those of the host.

During the lytic cycle, the HSV1 genes are transcribed and expressed in a highly regulated cascade in groups classified as immediate early (IE) or α, early (E) or β, and late (L) or γ relating to their time of expression from initial infection (Honess and Roizman 1974). The lytic gene cascade is illustrated in figure 1-3.

Transcription of the IE genes is stimulated by the HSV tegument protein VP16 (or vmw65), brought into the nucleus by host cell factor (HCF) along with the viral DNA (Batterson *et al.* 1983; La Boissiere *et al.* 1999). VP16 interacts with at least two cellular proteins to achieve this. Oct-1 is a POU domain transcription factor (Sturm *et al.* 1988) that recognises and binds to the TAAT region of the TAATGARAT regulatory elements found in each IE gene promoter. VP16 forms a complex with HCF and then binds to the Oct-1/TAATGARAT complex through the POU domain and the GARAT sequence (Gerster and Roeder 1988; Katan *et al.* 1990; Kristie and Sharp 1990; O'Hare *et al.* 1988; Preston *et al.* 1988; Stern and Herr 1991; Stringer *et al.* 1990; Xiao and Capone 1990). Recently the Oct-1 protein has been shown to only be required at a low multiplicity of infection (MOI) (Nogueira *et al.* 2004), suggesting that interactions of VP16 with other factors may also be important. A potent VP16 transcriptional activation domain then activates IE gene transcription, probably through interaction with TFIIB and TFIID (Gupta *et al.* 1996; Stringer *et al.* 1990; Triezenberg *et al.* 1988). Mutation of VP16 produces a virus greatly reduced in infectious properties (Ace *et al.* 1989). One other potential function of VP16 is its interaction with vhs, which it is

thought to downregulate and therefore prevent degradation of viral mRNAs (Lam *et al.* 1996; Matis *et al.* 2001; Mossman *et al.* 2000).

Transactivation by VP16 of IE gene transcription occurs without prior viral protein synthesis. There are 5 IE gene products – infected cell polypeptide (ICP) 0, 4, 22, 27 and 47. With the exception of ICP47, all of the IE gene transcripts regulate the expression of the remaining genes. Transcription of these and all subsequent viral genes is carried out by the host cell's RNA polymerase II (Alwine *et al.* 1974; Costanzo *et al.* 1977).

1.1.2.3.1 The IE genes

ICP0 is a multifunctional protein. It is not essential for viral replication, but ICP0 mutant viruses are impaired for growth at low multiplicities of infection (MOIs) (Sacks and Schaffer 1987; Stow and Stow 1986). It is a non-specific transactivator and can induce the expression of all classes of viral gene promoters as well as non-HSV promoters (Everett 1984; Gelman and Silverstein 1985; Nabel *et al.* 1988; O'Hare and Hayward 1985; Quinlan and Knipe 1985). ICP0 also interacts with ICP4, increasing activation of gene expression (Gelman and Silverstein 1986; Quinlan and Knipe 1985). ICP0 is a RING finger zinc-binding protein with E3 ubiquitin ligase activity which appears to target many cellular proteins for degradation via the proteasome pathway (Everett *et al.* 1999; Lomonte *et al.* 2001). ICP0 accumulates at PML bodies, also called ND10s, which is also where viral replication occurs, and causes their disaggregation (Everett and Maul 1994; Maul *et al.* 1993; Maul and Everett 1994). One other possible function of ICP0 is that is thought to be involved in inhibiting the host cell's interferon and antiviral responses early in infection (Eidson *et al.* 2002). ICP0 null viruses can infect cells, but the genomes then tend to become quiescent. This repression can be overcome by introducing ICP0 in *trans* (Harris *et al.* 1989; Samaniego *et al.* 1998; Zhu *et al.* 1990). ICP0 has a possible function in latency and reactivation because the gene is transcribed antisense to the LAT gene (see later) and viruses lacking ICP0 are

unable to reactivate (Halford and Schaffer 2001). The finding that ICP0 does not accumulate in the nucleus of neuronal cells may account for the preference to establish latency in these cells (Chen *et al.* 2000) and may be the reason that the 2kB LAT potentially encodes a protein which can substitute for its function (Thomas *et al.* 1999a).

ICP4 is an essential regulatory protein encoded by the R_S1 gene and is therefore present twice in the viral genome. It is necessary for the activation of both early and late gene expression (Watson and Clements 1980). It also functions to repress the expression of some viral genes, including itself, which it carries out via a direct binding to the respective gene promoters (DeLuca *et al.* 1985; O'Hare and Hayward 1985). The activation function is thought to occur via interaction with components of the basal transcription apparatus but not by direct binding. ICP4 can bind the HMG1 protein, which has the ability to cause DNA bending, and it is thought to then facilitate the interaction of ICP4 with TFIID (Carrozza and DeLuca 1998; Carrozza and DeLuca 1996; Grondin and DeLuca 2000; Gu *et al.* 1993; Gu *et al.* 1995; Gu and DeLuca 1994; Kuddus *et al.* 1995). Certain mutations in ICP4 effect early, but not late gene expression, suggesting that ICP4 may act differently on these promoters. Work has shown that this is in fact due to the interaction with different TFs or the Inr element, depending on whether the promoter is for an E or L gene (DeLuca *et al.* 1984; DeLuca and Schaffer 1988; Zabierowski and DeLuca 2004).

ICP27 is also essential for the replication of HSV1 and has multiple functions, both transcriptional and post-transcriptional. ICP27 is required for the progression of the expression cascade from E to L gene expression (McCarthy *et al.* 1989; Rice and Knipe 1990; Sacks *et al.* 1985) and transactivates a subset of early genes involved in DNA replication and also downregulates the expression of some IE and E genes via interaction with ICP4 and ICP0 (Hardwicke *et al.* 1989; McCarthy *et al.* 1989; McGregor *et al.* 1996; McMahan and Schaffer 1990; Rice and Knipe 1990; Sacks *et al.*

1985; Sekulovich *et al.* 1988; Uprichard and Knipe 1996). Post-transcriptionally ICP27 inhibits mRNA splicing, which gives an advantage to viral over cellular transcripts, as only four viral transcripts contain introns, and three of these are in IE genes which are spliced before maximal inhibition takes place (Hardy and Sandri-Goldin 1994). The inhibition has been shown to occur through the interaction of ICP27 with a cellular protein known to inhibit splicing (Bryant *et al.* 2000). Another important function of ICP27 is that it binds RNA and shuttles it to the cytoplasm from the nucleus, thus mediating the export of intronless viral mRNA (Mears and Rice 1998; Phelan *et al.* 1996; Sandri-Goldin 1998; Soliman *et al.* 1997).

ICP22 is not essential for viral replication in all situations (Sears *et al.* 1985), but it is necessary for the optimal expression of some late genes. ICP22 interacts with several cell cycle regulated proteins including cdc2 protein kinase that is activated in HSV-infected cells. Cdc2 kinase is involved in expression of the same set of late genes activated by ICP22 and thus possibly this function is mediated by the kinase (Advani *et al.* 2000). Another function of ICP22 is that it modifies the host RNA pol II by phosphorylating the large subunit. This generates a novel form that specifically represses host cell transcription (Long *et al.* 1999; Rice *et al.* 1995; Spencer *et al.* 1997). Finally, ICP22 also increases the stability and splicing of ICP0 mRNA (Carter and Roizman 1996).

ICP47 is non-essential for viral replication, but plays an important role in evading host immune responses. It does this by blocking presentation of viral peptides at the cell surface (Jackson and Peterson 1993). ICP47 binds to the transporter of antigen processing (TAP) complex and prevents peptide binding and thus translocation into the endoplasmic reticulum (ER) (Hill *et al.* 1995; Hill *et al.* 1994).

1.1.2.3.2 The E genes

Following IE gene expression, the E genes are transcribed. E gene products are primarily involved in viral DNA synthesis and encode proteins such as DNA binding proteins (e.g. ICP8, DNA polymerase, thymidine kinase (TK) and ribonucleotide reductase (RR)) as well as the ori binding proteins and the helicase/primase complex. When the E gene products are present in sufficient quantities, DNA replication can begin (Challberg 1986; Conley *et al.* 1981; Purifoy *et al.* 1977; Wu *et al.* 1988).

As mentioned, viral DNA replication occurs at ND10 structures in the nucleus and as synthesis progresses, the progeny DNA molecules accumulate in globular structures called replication compartments (Quinlan *et al.* 1984). DNA replication proceeds from the viral template, which as discussed in section 1.1.2.2, is thought to occur from a circular template by a rolling-circle mechanism producing concatemeric molecules. This is then cleaved and packaged into single units which make up the progeny genomes (Jacob *et al.* 1979).

1.1.2.3.3 The L genes

Concurrently with DNA replication, viral late genes start to be expressed, whilst early gene expression begins to subside. The majority of the late genes encode structural proteins of the capsid, tegument and envelope and on synthesis of these, progeny viral particles begin to assemble.

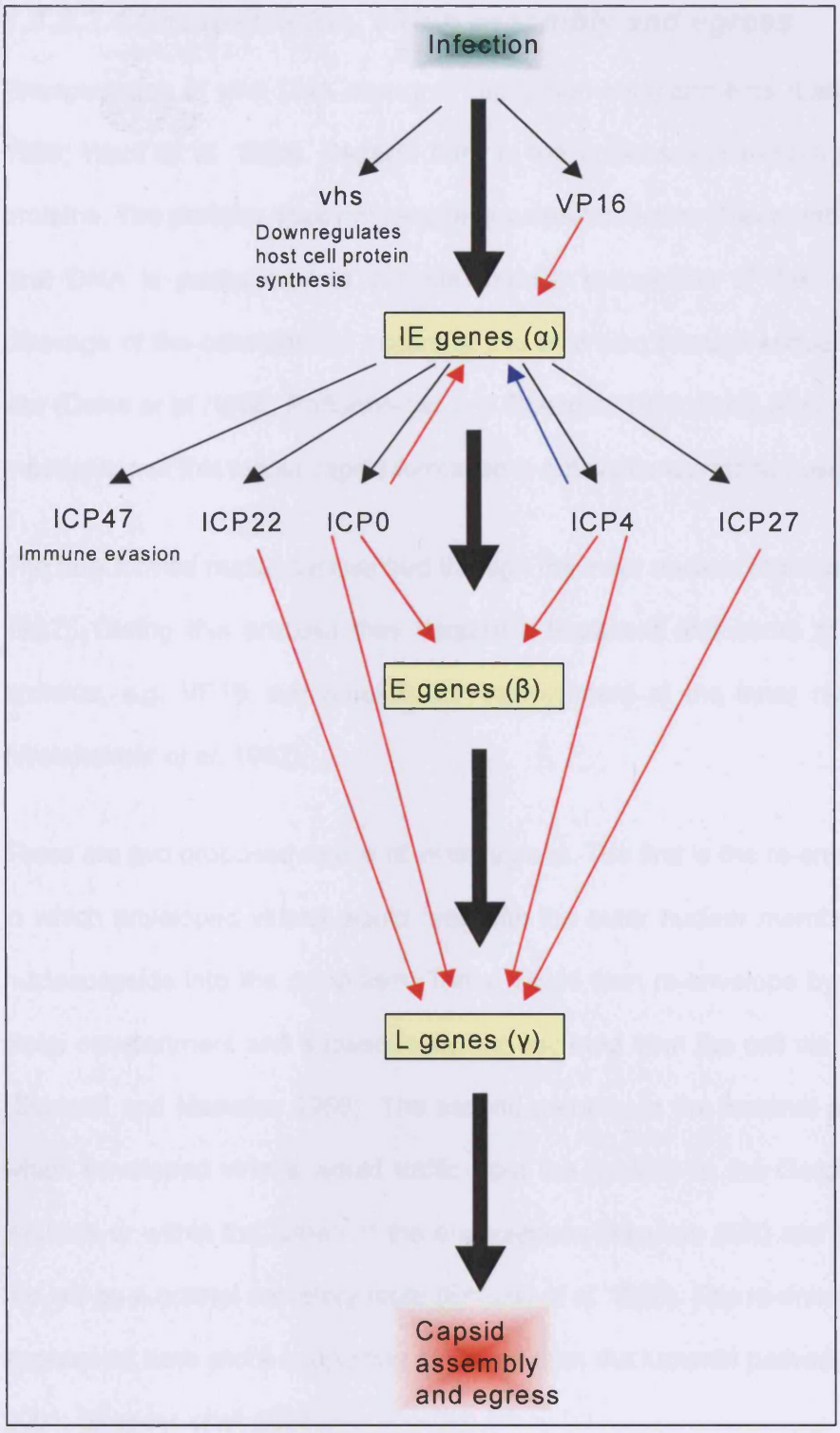


Figure 1-3 The HSV1 lytic gene regulatory cascade

Transcription of IE genes is transactivated by VP16. Expression of IE genes is required for expression of E and L genes. Known regulatory effects are marked in red (positive) and blue (repressive). There are a number of other interactions between IE genes that contribute to the regulation, which have been left off for purposes of clarity.

1.1.2.3.4 Encapsidation, virion assembly and egress

Encapsidation of viral DNA occurs in replication compartments (Lamberti and Weller 1998; Ward *et al.* 1996). Capsids form in the nucleus and require at least 7 capsid proteins. The process does not require any cellular factors (Newcomb *et al.* 1999). The viral DNA is packaged into capsids through recognition of the 'a' sequence and cleavage of the concatemers occurs at this time also through sequences at the same site (Deiss *et al.* 1986; Poffenberger and Roizman 1985; Stow *et al.* 1983). The exact mechanism of this nucleocapsid formation is not well understood however.

The fully formed nucleocapsids bud through the inner nuclear membrane (Vlazny *et al.* 1982). During this process they acquire a tegument and some of these tegument proteins, e.g. VP16, are required for envelopment at the inner nuclear membrane (Weinheimer *et al.* 1992).

There are two proposed routes of virion egress. The first is the re-envelopment model, in which enveloped virions would fuse with the outer nuclear membrane and release nucleocapsids into the cytoplasm. These would then re-envelope by budding into the Golgi compartment and subsequently are secreted from the cell via a vesicular route (Siminoff and Menefee 1966). The second pathway is the lumenal pathway model in which enveloped virions would traffic from the nucleus to the Golgi compartment in vesicles or within the lumen of the endoplasmic reticulum (ER) and be released from the cell by a normal secretory route (Enquist *et al.* 1998). The re-envelopment pathway appears to have more supporting evidence than the lumenal pathway (Granzow *et al.* 2001; Skepper *et al.* 2001).

Viral spread from cell to cell can occur through the extracellular space as well as through cell junctions. The gE and gI glycoproteins are required for this method of virus movement (Dingwell *et al.* 1994; Dingwell and Johnson 1998). In neurons, there is limited replication of HSV1; therefore the virus is transported from neuron to neuron

via the nerve terminals. This occurs more rapidly than to other non-neuronal cells (Kuypers and Ugolini 1990).

1.1.2.4 Latency

Following productive infection in the mucosal epithelium, HSV1 enters the innervating sensory neurons and is transported to the ganglia where it can establish a life-long latent state. During this time, no lytic-cycle genes are expressed and the genome is transcriptionally silent apart from the duplex LAT region, which produces RNA species called LATs. Periodic reactivation can occur, leading to lytic infections in the same or similar sites as the initial infection.

1.1.2.4.1 Establishment and maintenance of latency

It is thought that the outcome of infection is determined early on and that the lytic cycle and latency can reflect distinct pathways. During the first few days following mouse footpad infection, neurons of the dorsal root ganglia (DRG) either produce lytic cycle proteins or LATs but not both (Margolis *et al.* 1992). Where limited replication of virus is supported in sensory neurons (Knotts *et al.* 1974; Kramer *et al.* 1998), this is eventually turned off (or in some cases to a very low level) and the genomes tend to turn quiescent without producing LATs. These differences in the establishment of latency have lead to a number of theories as to by which mechanism it occurs.

There is much evidence to show that latency can be established without prior lytic gene expression. An ICP4- virus cannot express E or L proteins but has been shown to establish latency (Dobson *et al.* 1990; Katz *et al.* 1990; Sedarati *et al.* 1993) and also the *in*1814 virus which has a mutation in VP16 and so prevents transactivation of the IE genes (Ace *et al.* 1989) can establish latency as efficiently as wild-type virus (Ecob-Prince *et al.* 1993; Steiner *et al.* 1990; Valyi-Nagy *et al.* 1991; Valyi-Nagy *et al.* 1992).

It was suggested that factors present in neurons but not other cells might lead to this block in replication. One possibility is based on the suggestion that in cells which are not permissive for a lytic infection, splice variants of the POU domain protein Oct-2 compete with Oct-1 for the TAATGARAT binding site (Lillycrop *et al.* 1991). These isoforms cannot interact with VP16 and so transactivation of the IE gene promoters does not ensue (Lillycrop *et al.* 1993). It was reported that when the Oct-2 splice variants were transfected into cells that were permissive for HSV1 replication, IE gene expression was dramatically reduced. It was proposed that this would then abort the lytic infection at an early stage and force the virus into latency. In support of this, Oct-1 is a ubiquitous cellular protein whereas the Oct-2 isoforms are only found in neurons, consistent with the primary sites of establishment of lytic and latent infections, respectively. However, there have been failed attempts to reproduce these findings and there has been some controversy over whether the type of neurons capable of harbouring a latent infection actually express Oct-2 (Hagmann *et al.* 1995; Turner *et al.* 1996). Therefore, if Oct-2 is involved with the repression of IE gene transcription, it may not be the only factor and other POU-domain proteins found in neurons have also been implicated (Hagmann *et al.* 1995; Turner *et al.* 1997).

Other possibilities for the block to IE gene transcription include the observation that HCF is distributed differently in neuronal and non-neuronal cells. In neurons, HCF is found in the cytoplasm, whereas in most other cell-types it is found in the nucleus (Kristie *et al.* 1995; Kristie *et al.* 1999). It is therefore possible that in neurons VP16 is not transported to the nucleus and consequently IE genes do not become transcribed (La Boissiere *et al.* 1999). However, experiments which have shown that latency can be established even in the presence of functional VP16 suggest that this may not be the whole answer either (Sears *et al.* 1991).

On establishment of latency, the infected cell can harbour up to several hundred copies of the viral genome (Efstathiou *et al.* 1986; Hill *et al.* 1996b; Hill *et al.* 1996a; Rock and Fraser 1983), although the number appears to be virus-strain dependent (Sawtell *et al.* 1998; Sawtell 1998). The number of harboured genomes appears to be maintained consistently throughout latency (Sedarati *et al.* 1989; Sedarati *et al.* 1993) as does the number of genomes expressing LATs (Hill *et al.* 1996a). However, it has been shown by *in situ* hybridisation that the number of neurons containing HSV1 DNA is 2-3 fold greater than the number of LAT-expressing neurons (Mehta *et al.* 1995). The LATs and their function in the maintenance of latency and reactivation will be discussed in more detail later.

1.1.2.4.2 Reactivation

During a clinical latent infection, a subset of the neurons containing latent genomes can reactivate, whereby infectious virus is carried in an anterograde fashion by axonal transport to peripheral sites of original infection (Colberg-Poley *et al.* 1981).

Reactivation can be caused by physical or psychological stress in humans (Young *et al.* 1976). In animal models, reactivation stimuli include neurectomy (Walz *et al.* 1974), chemical stimuli (Harbour *et al.* 1983), UV irradiation (Spurney and Rosenthal 1972), mild trauma (Hill *et al.* 1978) and transient hyperthermia (Sawtell and Thompson 1992).

It is likely that viral replication during reactivation involves a different pathway to initial replication on infection, as VP16 is not present in latently-infected cells, is a late gene, is not necessarily able to translocate to the nucleus and is not sufficient to stimulate reactivation (La Boissiere *et al.* 1999; Sears *et al.* 1991). It was shown that the first events leading to viral replication in reactivating neurons are initiation of DNA replication (Nichol *et al.* 1996) and expression of E genes (Tal-Singer *et al.* 1997), although there is also evidence that neuronal damage or other stimuli possibly activate

cellular transcription factors (TFs) that substitute for VP16 and/or IE proteins to initially target the ICP0 promoter, resulting in expression of ICP0 (Loiacono *et al.* 2003; Tal-Singer *et al.* 1998).

As mentioned, it has been shown that the genome copy number in latently infected cells is virus-strain specific and that this relates to the ability to reactivate *in vivo* and the probability of doing so (Sawtell *et al.* 1998; Sawtell 1998). Deleting the same 371bp of the LAT region from two different virus strains and obtaining two different reactivation phenotypes illustrated this (Loutsch *et al.* 1999).

Various other deletions from viral genomes have led to an understanding of which genes are required for reactivation. A TK-null virus was able to establish a latent infection, but was unable to reactivate (Coen *et al.* 1989). ICP0 is also not required for establishment of latency, but again is needed for reactivation (Cai *et al.* 1993; Clements and Stow 1989; Gordon *et al.* 1990; Leib *et al.* 1989), which lends further weight to the hypothesis that it is the ICP0 promoter that is the first target in the reactivation process.

Viruses deleted in the LAT region are able to establish a latent infection, although it is thought more poorly than LAT-positive viruses (Izumi *et al.* 1989; Perng *et al.* 2000b; Perng *et al.* 2000a; Sedarati *et al.* 1989; Thompson and Sawtell 1997). Reactivation also does not absolutely depend on the presence of LAT in the genome (Block *et al.* 1990; Ho and Mocarski 1989), but LAT-negative viruses reactivate with reduced frequency than wild-type viruses and not spontaneously (Block *et al.* 1993; Leib *et al.* 1989; Perng *et al.* 1994). The spontaneous reactivation function of LAT in the rabbit model has been localised to a 348bp region within the first 1.5kb of the 8.3kb primary transcript (see below) (Bloom *et al.* 1996; Perng *et al.* 1996). It also appears that not all viruses that produce LATs reactivate (Ecob-Prince and Hassan 1994). Thus the

exact function of the LAT region in the establishment, maintenance and reactivation of the HSV genome is not completely understood.

1.1.2.4.3 The LATs

The LATs are a family of RNA transcripts, originating from the complementary DNA strand to ICP0. They were first identified in latently infected sensory neurons by *in situ* hybridisation of murine ganglia and by northern blot (Stevens *et al.* 1987). They were also detected in rabbits and humans at around the same time (Croen *et al.* 1987; Rock *et al.* 1987; Spivack and Fraser 1987).

The LATs are generally thought to be the only genes to be transcribed during latency, although very low level expression of other genes has been shown during this time by PCR, including ICP4 and TK (Kramer and Coen 1995).

There are 3 species of LAT – colinear transcripts of a minor 8.3kb species and two major species; one of 2kb that is spliced from the 8.3kb and another of 1.5kb that is further spliced from the 2kb LAT (Spivack and Fraser 1987; Wechsler *et al.* 1988b; Wechsler *et al.* 1988a). These are illustrated in figure 1-4. The 8.3kb transcript is only found during lytic infection and is polyadenylated (Dobson *et al.* 1989; Zwaagstra *et al.* 1990). The two smaller LATs are thought to be non-polyadenylated stable introns with a lariat structure. The unusual stability is attributed to a non-conventional splice-junction that is not recognised by cellular degradation factors (Krummenacher *et al.* 1997; Mukerjee *et al.* 2004; Thomas *et al.* 2002a; Zabolotny *et al.* 1997). The 2kb LAT is produced during lytic infection and then a further splicing event occurs when latency has been established to form the 1.5kb LAT (Alvira *et al.* 1999).

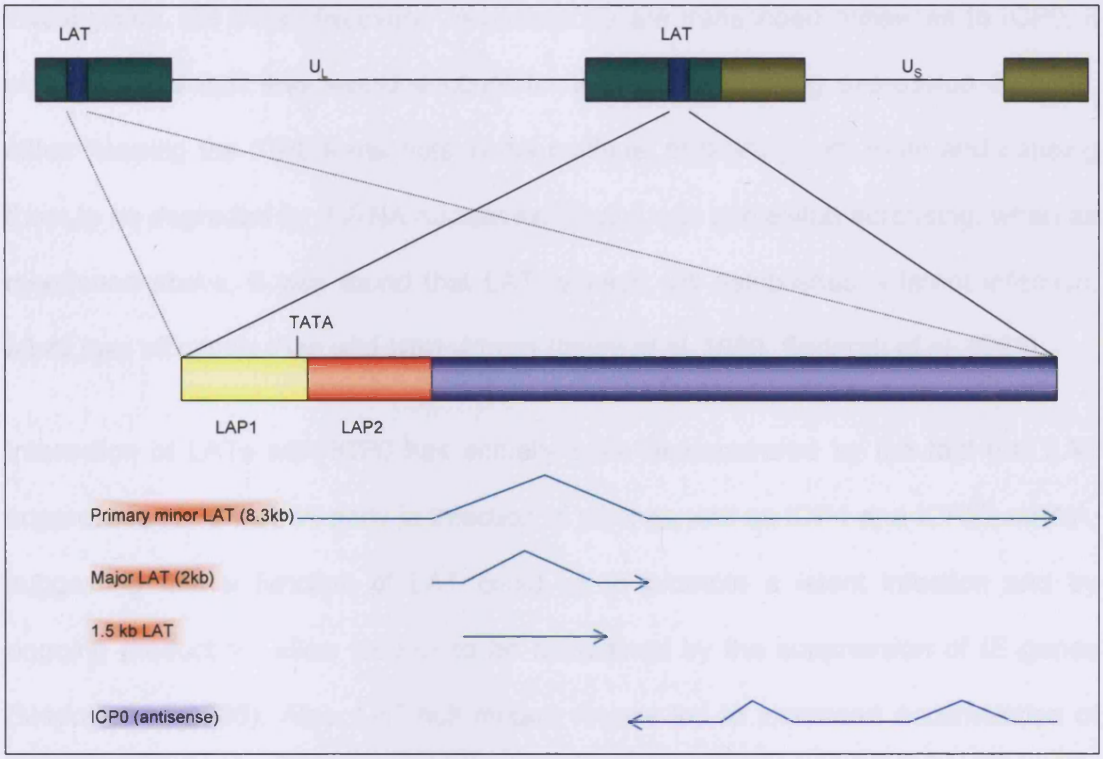


Figure 1-4 Organisation of the LAT region

The LAT region is located in the long repeat regions of the genome, thus is present in two copies as indicated. The primary LAT is an unstable 8.3kb transcript. The major species are the smaller spliced LATs of 1.5 and 2 kb. The LATs are transcribed antisense and partially complementary to the gene for ICP0. The two latency-associated promoters are also shown (see section 1.1.2.3.4).

Since the LATs are the most abundant transcripts detected during latency it would seem reasonable to assume they have some role in the establishment of latency, and/or in maintenance and/or reactivation.

Despite this fact, the exact function of LATs still remains elusive after much investigation. On initial discovery, because they are transcribed antisense to ICP0, it was assumed that they would account for latency by blocking expression of ICP0, either keeping the ICP0 transcripts in the nucleus, or binding with them and causing them to be degraded by dsRNA nucleases. Thus it was somewhat surprising, when as mentioned above, it was found that LAT- viruses still established a latent infection, albeit less efficiently than wild-type viruses (Izumi *et al.* 1989; Sedarati *et al.* 1989).

Interaction of LATs with ICP0 has actually been demonstrated by the fact that LAT suppresses ICP0 mRNA early in infection *in vitro*, as well as ICP4 and ICP27 mRNA, suggesting that a function of LAT could be to promote a latent infection and by ongoing production, allow latency to be maintained by the suppression of IE genes (Mador *et al.* 1998). Also, LAT-null mutant viruses led to increased accumulation of ICP0 RNA *in vitro* (Arthur *et al.* 1998), reinforcing the argument that LATs suppress lytic gene expression. It was suggested that this block could be overcome upon reactivation by the E genes being expressed without the prior expression of the IE genes (Tal-Singer *et al.* 1997). More recently, however, it was demonstrated that a direct antisense mechanism between the 2 kb LAT and ICP0 does not appear to occur in non-neuronal cell lines (Burton *et al.* 2003). These authors suggest that LAT effects are cell type specific and that it is unlikely that an antisense mechanism is mediated by the 2 kb LAT *in vivo*.

It has been shown that during latency the 2kb LAT is found in the nucleus, but in lytic infection, it can also be detected in the cytoplasm, where it interacts with ribosomal proteins, suggesting that perhaps LAT transcripts have a role in affecting the

translational machinery or have a structural role in the ribosomal complex, perhaps serving to downregulate translation of IE genes and turn the virus towards a latent state (Ahmed and Fraser 2001; Goldenberg *et al.* 1997; Nicosia *et al.* 1994; Spivack and Fraser 1987; Stevens *et al.* 1987).

The observation of several ORFs within the LAT region has led to the suggestion that LAT might encode a protein that functions during latency (Lagunoff and Roizman 1994). Furthermore, the related α-herpesvirus bovine herpes virus 1 (BHV1) produces a protein from within its LAT region, which possibly functions to prevent death of latently infected neurons (Schang *et al.* 1996). However, the fact that the HSV LAT transcripts are intranuclear during latency has not led to a great deal of support for this idea. Despite this, conserved ORFs have been found in the major 2kb LAT and deregulated expression of the largest of these has been shown to significantly enhance the growth of viruses deficient in IE genes and most notably ICP0 (Thomas *et al.* 1999a). A myc-tagged version of this protein also colocalised to ND10 structures *in vivo* (Thomas *et al.* 2002b). This led the authors to propose a model whereby the LAT RNA acts as an antisense transcript to ICP0 during the establishment and maintenance of latency but expresses a protein during reactivation, compensating for the lack of ICP0 and other IE genes and thus allowing the lytic cycle to proceed. However, because if this protein were expressed, it would be tightly regulated and only be very rarely expressed, it has proved difficult to detect. As well as this, mutation of the LAT ORFs has failed to produce a phenotypically changed latency profile in animal models and thus this work has proved controversial.

A further role suggested for LAT is in keeping the latently infected neurons alive, either by dampening toxic IE gene expression or by preventing apoptosis.

Reducing IE gene expression as a means of promoting neuronal survival was first suggested as a function of LAT by groups investigating LAT *in vivo* (Chen *et al.* 1997;

Garber *et al.* 1997; Perng *et al.* 1999). It was observed that the virulence of the virus increased when the LAT gene was deleted and increased IE levels were seen. Other work carried out *in vitro* also gave weight to the theory that this was effected by LATs (Farrell *et al.* 1991; Mador *et al.* 1998).

There is also a fair amount of data showing LAT to be involved in preventing apoptosis, or programmed cell death. Apoptosis is a mechanism used by infected cells to prevent the spread of foreign infection, such as that of a virus. Therefore, viruses have had to evolve the ability to prevent this to ensure their survival.

An anti-apoptotic effect of LAT was first shown using plasmids containing the first 1.5kb of the 8.3kb LAT in neuronal and non-neuronal cells *in vitro.* This 1.5kb sequence protected cells from apoptosis when induced chemically (Inman *et al.* 2001). Further similar plasmid studies with different parts of the LAT region confirmed this and localised the region required for the apoptotic function to two separate areas of the first 1.5kb of the 8.3kb RNA (Ahmed *et al.* 2002; Henderson *et al.* 2002; Jin *et al.* 2003). Recently, the apoptotic function has been tested *in vivo* and localised to the LAP1 promoter and the first 1.5kb of the 8.3kb LAT (Branco and Fraser 2005). Furthermore, replacement of this region with an unrelated anti-apoptotic gene showed a similar prevention of neuronal death *in vivo* (Jin *et al.* 2005).

With respect to all of the possible functions ascribed to the LATs, it is likely that no one individual aspect provides the whole picture, but that each contributes somehow to the workings of this multi-functional region.

1.1.2.3.4 The latency promoters

There are two promoters involved in the production of LATs. These are named latency – active promoter 1 (LAP1) and latency – active promoter 2 (LAP2) and their different roles will be discussed below. Their position in the LAT region is shown in figure 1-4.

1.1.2.4.4.1 LAP1

LAP1 was the first LAT promoter to be identified. It was mapped to a region >660bp upstream of the major 2kb LAT start site and confirmed as the promoter responsible for expression of the LATs during lytic infection of cultured neuronal and non-neuronal cells (Zwaagstra *et al.* 1989; Zwaagstra *et al.* 1990).

LAP1 contains classic RNA pol II transcriptional regulatory sites, including a TATA box, Sp1 sites and a CAAT element (Wechsler *et al.* 1989; Zwaagstra *et al.* 1989). Primer extension analyses identified transcription initiation as coming from a location 28 nucleotides downstream of the TATA box (Zwaagstra *et al.* 1990). The constitutive basal activity was mapped to a region –2 to –161bp upstream of the initiation site (Zwaagstra *et al.* 1991).

It was discovered that deletion of the promoter region causes an absence of LATs in mice during latency (Mitchell *et al.* 1990). This was narrowed down to the region around the TATA box, which has been shown to be crucial for LAT expression during latency. A slight mutation of the TATA box causes a severe reduction in LAT expression (Nicosia *et al.* 1993; Rader *et al.* 1993; Soares *et al.* 1996).

Many studies have identified *cis*-acting elements within LAP1 that contribute to efficient transcription. CAT assays in neuronal and non-neuronal cell lines showed that LAP1 has neuronal specificity (Batchelor and O'Hare 1990; Devi-Rao *et al.* 1991; Morrow and Rixon 1994; Zwaagstra *et al.* 1990). Specifically, a LAT promoter binding factor (LBPF) sequence was identified approximately 64bp upstream of the transcription start site. Deletion of this element resulted in 30 times less activity of LAP1 (Zwaagstra *et al.* 1991). It was subsequently found that upstream stimulatory factor (USF) proteins can bind to the LBPF sequence and therefore these may be involved in the activation at this element (Kenny *et al.* 1997).

It was also shown that LAT expression in neurons is specifically regulated during latency and it was proposed that in the absence of viral proteins this must be actioned by cellular factors. In neuronal cells, LAP1 is activated by nerve growth factor (NGF) and sodium butyrate through the Ras/Raf pathway in several regions of the promoter; thus this is a potential mechanism through which the promoter is activated during latency (Frazier *et al.* 1996a; Frazier *et al.* 1996b).

LAP1 also contains two cAMP response element (CRE)-like elements; CRE-1 and CRE-2 (Kenny *et al.* 1994; Leib *et al.* 1991). It was shown that LAP1 is able to bind cAMP and respond to it; therefore it was proposed that this has a potential role in reactivation (Leib *et al.* 1991). Further investigation concluded that mutation of CRE-1 does cause a reduction in induced and spontaneous reactivation *in vivo* (Bloom *et al.* 1997). Both CRE-like elements have also been shown to bind members of both ATF/CREB and AP-1 factors, suggesting that other cellular pathways could be involved in regulation of LAP1 (Kenny *et al.* 1994; Millhouse *et al.* 1998).

Additional cellular factors implicated in LAP1 regulation are the early growth response factor (EGR) family of TFs. These are inducible TFs that have been shown to inhibit LAP1 activity *in vitro* by binding to an element immediately downstream of the TATA box. EGR was shown to compete with and displace TATA binding proteins (TBPs) at the start site to prevent transcription (Chiang *et al.* 1996; Tatarowicz *et al.* 1997). Despite this, the absence of detectable EGR in trigeminal ganglia has thrown doubt onto the relevance of this finding.

Regulation of LAP1 by HSV1 proteins has been investigated and it was shown that ICP0 can up-regulate activity in neuronal cells by binding to the promoter (Batchelor and O'Hare 1992). Conversely, ICP4 downregulates LAP1 during productive infection by binding to a sequence near the transcription start site, in the same way that it downregulates its own promoter (Farrell *et al.* 1994). Furthermore, ICP4 inhibits

activation by USF at the transcription start site and therefore must interact with the general transcription complex to cause repression (Rivera-Gonzalez *et al.* 1994). The exact implications of these interactions are not properly understood, but it would seem that the IE proteins may act upon LAP1 in order to maintain LAT expression at an appropriate level during lytic expression and the establishment of latency.

The discovery that deletion of LAP1 did not prevent the production of LATs during lytic infection led to the proposal that another LAT promoter may exist (Nicosia *et al.* 1993).

1.1.2.4.4.2 LAP2

Two fairly extensive studies set about identifying the location and nature of the putative second LAT promoter of HSV-1. Goins *et al* (Goins *et al.* 1994) tested the promoter activity of constructs consisting of regions downstream of LAP1 with varying deletions, in CAT assays. The region lacks a TATA box, but does have functional promoter activity. This was thus named LAP2. The region of LAP2 allowing maximal CAT expression in their assay contains an Sp1 site, putative AP2 and E2F sites. The basal promoter activity contained homology to the initiator element found in other TATA-less housekeeping gene promoters. Goins *et al* identified a region that modulates the basal activity of LAP2, which contains an AP2 site and a CT rich element, consisting of a core sequence often found within the promoters of many cellular RNA polymerase II housekeeping genes. These atypical promoters are very GC rich, which it was predicted might attract TFs and/or alter nucleosome structure. Such promoters also often possess upstream TATA box elements similar to the situation with LAP2.

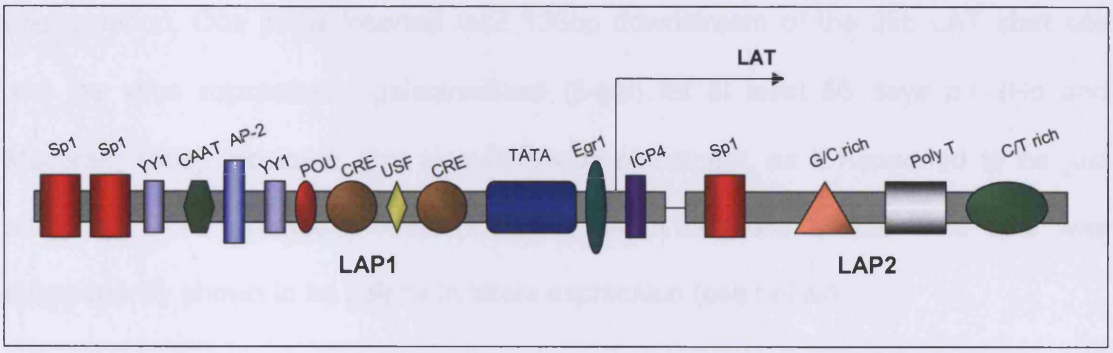
LAP2 was shown to only work in the forward direction and Goins *et al* found that when LAP1 and LAP2 were present, LAP1 reduced the activity from LAP2 in transient assays. ICP4 was able to reduce the basal LAP2 activity despite absence of a binding site. It was therefore proposed that ICP4 possibly interferes with and prevents TFs

from binding to LAP2. On testing the promoter activity of LAP2 *in vivo*, it was found that LAP2 is only active at lytic times (Goins *et al.* 1994). It was suggested that LAP1 and LAP2 might not be independent latency promoters but form part of a complex promoter-regulatory region.

Chen *et al* (Chen *et al.* 1995) used deletions of LAP1 and/or LAP2 in viruses to examine the relative contributions of each promoter during lytic infection *in vitro* and during latent infection *in vivo.* They determined that LAP2 is predominantly responsible for 2kb LAT production during acute infection and that this is dependant upon viral DNA replication. During latency, LAP1 was primarily responsible for 2kb LAT accumulation, LAP2 alone producing no LAT. The production of LAT by LAP1 was increased by the presence of LAP2 however, suggesting that LAP2 may play a role during latency other than as a promoter.

One other group investigated the role of LAP2 in HSV2 (Yoshikawa *et al.* 1996). They found less sequence homology between LAP2 than LAP1 as compared to HSV1. They observed that deleting the LAP2 promoter diminished LAT production in lytic and latent infections and that LAP2 could not produce LATs alone during latency. This concurred with the findings with HSV1 LAP2, the only difference being that LAP2 appeared to have no influence on LAT expression during latency from LAP1.

The two LAT promoters and their constitutive elements are illustrated in figure 1-5.



Adapted from (Jones 2003)

Figure 1-5 Schematic representation of the two LAT promoters

The LAT promoters contain numerous *cis*-acting elements that can be bound by cellular transcription factors or viral proteins. LAP2 contains elements similar to TATA-less housekeeping gene promoters.

1.1.3 Long-term expression from the LAT region

The knowledge that HSV1 remains latent for the lifetime of the host, with the majority of the genome shut-down and just the LAT region transcriptionally active, led to a search to find the mechanism by which the virus achieves this. The ability to harness this mechanism and use it to enable continued transgene expression is desirable for use in gene therapy applications.

Most groups researching this area used the *lacZ* gene linked to the latency promoters in order to ascertain which elements of the LAT region were responsible for the phenomenon. One study inserted *lacZ* 136bp downstream of the 2kb LAT start site and the virus expressed β-galactosidase (β-gal) for at least 56 days p.i. (Ho and Mocarski 1989). However, this was fortuitous placement, as it happened to be just after the LAP2 promoter, which had not been identified at the time and was subsequently shown to be helpful in latent expression (see below).

However some early experiments, before the identification of the LAT promoters, simply inserted *lacZ* linked to an endogenous promoter into the virus at various *loci* and looked for latent expression. Most of these resulted in no latent expression, although one, in which the murine moloney leukaemia virus (MMLV) LTR promoter was linked to *lacZ* and inserted into ICP4, expressed β-gal for at least 24 weeks in mouse sensory neurons (Dobson *et al.* 1990). It has since been suggested that this was seen due to the proximity of the insertion to the LAT region, which allowed the continued expression. This arrangement was also used in a virus injected into the hippocampus. Again, β-gal was seen expressed for up to 6 months, although it was reported that the expression was very low and could only be detected by PCR (Bloom *et al.* 1995).

Before the discovery of LAP2, *lacZ* and NGF were used to replace 1.5kb downstream of the LAP1 TATA box and although both genes were expressed highly in lytic infection, they were turned off during latency, suggesting that other elements were required than just LAP1 for latent expression (Coffin *et al.* 1996; Margolis *et al.* 1993). This shut-off of expression was also found using deletion mutants of LAP1 driving *lacZ* (Dobson *et al.* 1995).

As use of the MMLV LTR had been previously successful in driving long-term expression, this was linked to LAP1 driving *lacZ* expression in the gC gene of HSV1 (Lokensgard *et al.* 1994). This construct was shown to express *lacZ* during latency, whereas both promoters alone did not. Therefore, the authors suggested that a region similar to the MMLV LTR must exist in HSV1 to allow LAP1 to remain active, perhaps by maintaining an open nucleosomal structure of the region. The MMLV LTR was again shown to allow a long-term function when linked to LAP1 by inserting it upstream and in the reverse orientation to LAP1 and driving *lacZ*. This different arrangement of the promoters still allowed latent expression in mice 18 months after injection (Carpenter and Stevens 1996).

On identifying the LAP2 promoter, this was linked to *lacZ* and inserted into the gC locus and shown to express β-gal in the TG of infected mice during latency, although expression was weak (Goins *et al.* 1994).

Following discovery of the LAP2 promoter, several groups looked at the long-term expression given when leaving both promoters intact in the virus. One approach was to insert an internal ribosome entry site (IRES) after all recognised LAT regulatory sequences at the time, thus allowing expression of *lacZ* from the LAT region (Lachmann and Efstathiou 1997). This virus did not express β-gal in cultured cells, but when injected into mice, staining of sensory ganglia increased with the onset of latency and persisted, suggesting that the reporter gene was under true latent

promoter control. This virus was also used to investigate long-term expression in the CNS (Smith *et al.* 2000). The authors reported that the virus gave latent expression in motor neurons for longer than other types of cells (up to 1 year), suggesting that the LAT promoter region may have a preference for these types of neurons during latency.

Another group used an element consisting of 1.1kb downstream of LAP1, encompassing LAP2 plus another approximately 500bp downstream, named the long-term expression element (LTE). This was placed adjacent to LAP1 in both orientations and then used to drive *lacZ* expression by placing the reporter gene within an intron (Lokensgard *et al.* 1997). These cassettes were introduced into the virus at the gC gene. The LTE allowed long-term expression of β-gal in murine ganglia in both directions, but was most effective when in the forward orientation and downstream of LAP1.

The same group performed further investigations with the LTE. They constructed cassettes that linked the LTE in both orientations to TK and CMV promoters and compared them to the LTE linked to LAP1, in transient assays. They also made equivalent viruses of the LAP1-LTE arrangement (with the cassettes in gC) and tested them *in vitro* and *in vivo* (Berthomme *et al.* 2000). They showed that the LTE region contains enhancer activity in a non cell-type specific and non promoter-type specific fashion. In a virus *in vitro*, the LTE increased expression from LAP1 only when in the forward orientation, which they suggested could be promoter activity using a TATA box present just before the 3' end of the LTE that could be activated by ICP4. *In vivo,* the LTE increased expression from LAP1 during lytic and latent infection. The LTE functioned in both directions, although the forward direction enhanced activity better than the reverse. The authors suggested that LAP2 has promoter activity during acute infection, but that the enhancer activity of the region takes over during latency.

The LTE was also tested in our laboratory for its ability to confer long-term activity onto exogenous promoters (Palmer *et al.* 2000). An equivalent region downstream of LAP1, named LATP2, was linked to a minimal NSE promoter, the CMV promoter and the MMLV LTR in the LAT region and in other loci of HSV1 and showed an ability to confer long-term expression onto all of these promoters when tested in mice *in vivo*, by the presence of β-gal during latent timepoints in infected ganglia. The authors suggested that LATP2 might be able to locally remodel the viral chromatin to allow continued expression during latency, such that exogenous promoters placed adjacent to the region took on this open conformation and remained active.

The arrangement of the LAT region promoters and regulatory elements, as well as the ability of various arrangements of the region to direct long-term expression is illustrated in figure 1-6.

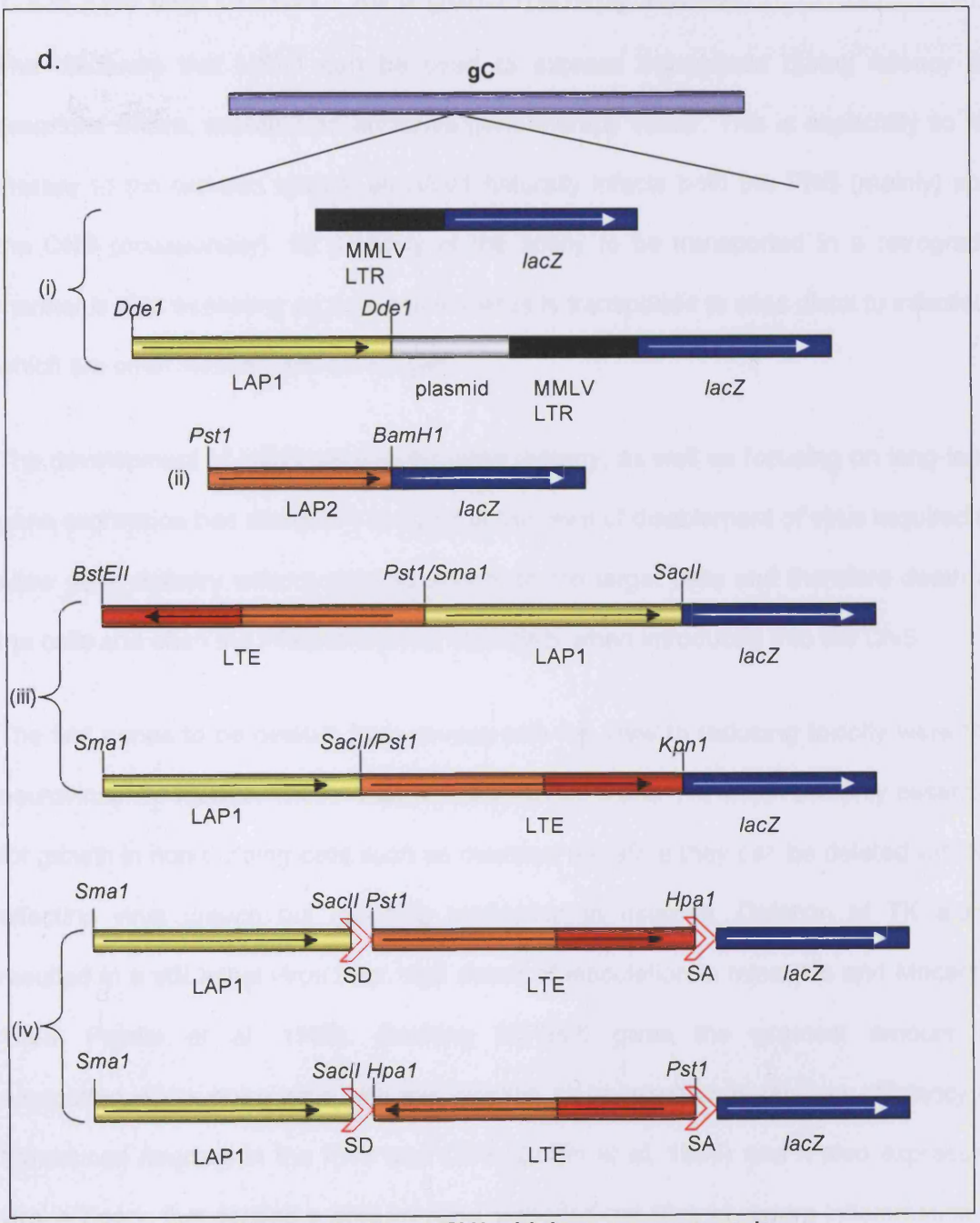
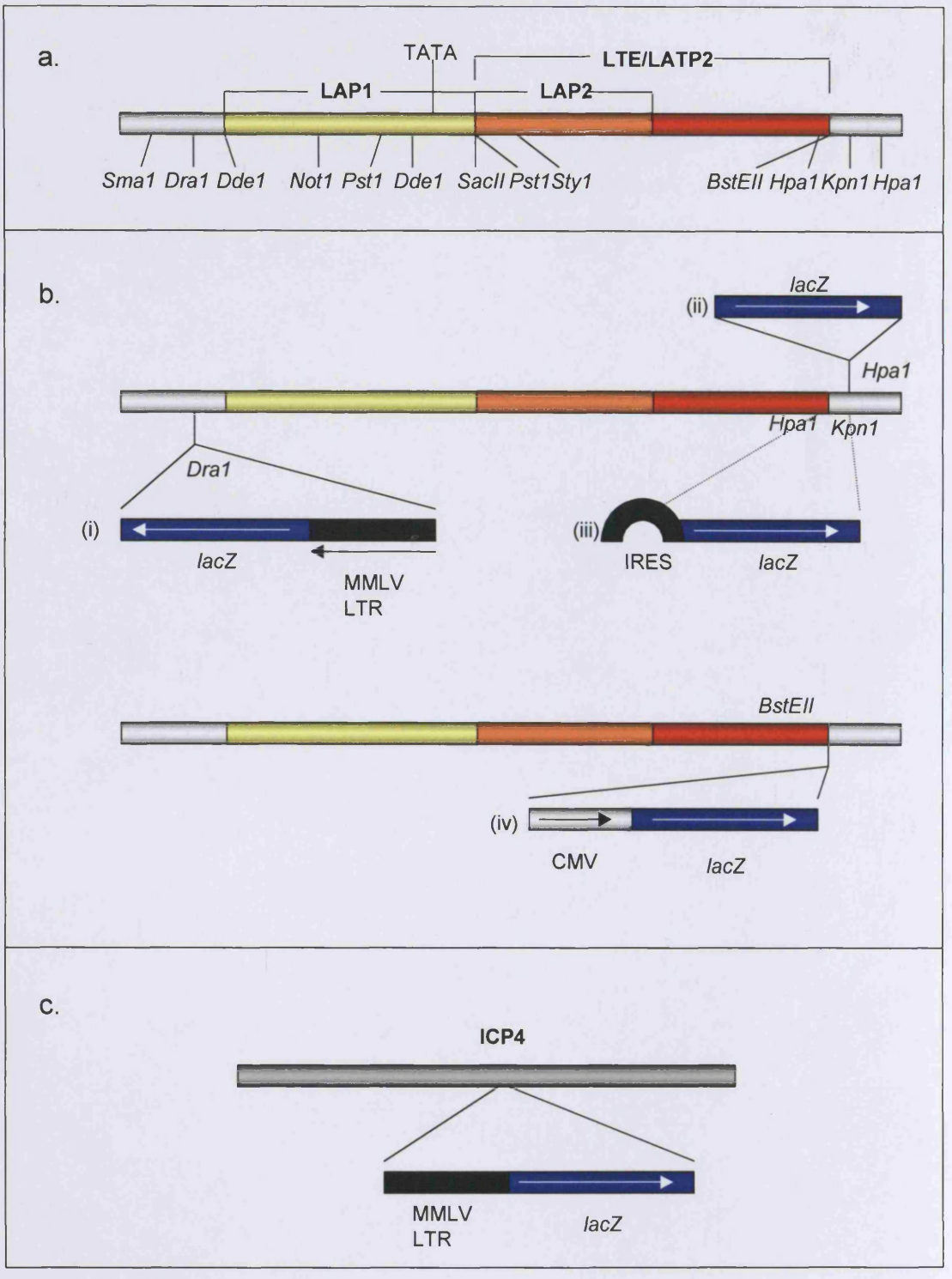


Figure 1-6 Cassettes used within HSV1 with long-term expression

a. Diagram of the LAT region showing the LAT promoters and the LTE/LATP2. Significant restriction sites have been marked.
b. Long-term cassettes that have been inserted into the LAT region.
c. Long-term cassette used in ICP4.
d. Long-term cassettes that have been inserted into the gC gene. (SA and SD = splice acceptor and splice donor respectively).

References as follows:
b(i): (Carpenter and Stevens 1996); b(ii): (Ho and Mocarski 1989); b(iii): (Lachmann and Efstathiou 1997), (Smith *et al.* 2000);b(iv): (Palmer *et al.* 2000).
c: (Dobson *et al.* 1990).
d(i) : (Lokensgard *et al.* 1994): d(ii) : (Goins *et al.* 1994); d(iii) : (Lokensgard *et al.* 1997); d(iv) : (Berthomme *et al.* 2000).


a.
TATA
LTE/LATP2
LAP1
LAP2
Sma1 Dra1 Dde1 Not1 Pst1 Dde1 SacII Pst1Sty1 BstEII Hpa1 Kpn1 Hpa1
b.
lacZ
(ii)
Hpa1
Hpa1 Kpn1
Dra1
(i)
lacZ
MMLV
LTR
(iii)
IRES
lacZ
BstEII
(iv)
CMV
lacZ
c.
ICP4
MMLV
LTR
lacZ

1.1.4 The use of HSV1 as a gene therapy vector

The discovery that HSV1 can be used to express transgenes during latency as described above, makes it an attractive gene therapy vector. This is especially so for therapy to the nervous system as HSV1 naturally infects both the PNS (mainly) and the CNS (occasionally). Its property of the ability to be transported in a retrograde manner is also appealing as it means the virus is transported to sites distal to infection, which are often inaccessible for surgery.

The development of HSV1 vectors for gene therapy, as well as focusing on long-term gene expression has also been directed at the level of disablement of virus required to allow gene delivery without causing toxicity to the target cells and therefore death of the cells and often the infected animal, especially when introduced into the CNS.

The first genes to be deleted from viruses with the view to reducing toxicity were the neurovirulence factors. These include ICP6, ICP34.5 and TK, which are only essential for growth in non-dividing cells such as neurons; therefore they can be deleted without affecting virus growth but reducing replication in neurons. Deletion of TK alone resulted in a still lethal virus after high doses of inoculation in mice (Ho and Mocarski 1988; Palella *et al.* 1988). Deleting ICP34.5 gives the greatest amount of neuroattenuation, but a virus with this deletion alone resulted in very low efficiency of transduced neurons in the PNS and CNS (Coffin *et al.* 1996) and it also expressed viral antigens that caused a host immune response resulting in severe inflammation in the brain (McMenamin *et al.* 1998a; McMenamin *et al.* 1998b).

This showed that further-disabled viruses would be required for successful gene delivery vectors. This was achieved by deleting IE genes. ICP4- viruses have significantly reduced gene expression (Chiocca *et al.* 1990; DeLuca *et al.* 1985; Dobson *et al.* 1990; Johnson *et al.* 1992a; Johnson *et al.* 1992b). However, the remaining IE genes are still expressed and when this virus was injected into the brain,

although this showed reduced virulence compared to wild-type, it still resulted in significant tissue damage (Chiocca *et al.* 1990). Other IE genes were deleted, including those for ICP0 or ICP27, or combinations of genes for ICP4/ICP22 and ICP4/ICP47 (Johnson *et al.* 1992a). These viruses however were still found to be toxic to neurons. Triple deletions of genes for ICP4, ICP22 and ICP27 were found to be significantly less toxic than other combinations of deletions (Krisky *et al.* 1998; Wu *et al.* 1996). Deleting all the IE genes from a virus resulted in infected cells that looked indistinguishable from non-infected cells (Samaniego *et al.* 1998). However, these viruses require complementing cell lines to grow that provide all of the IE genes, which are very toxic and as such the cell lines are only usable up to about passage 15 (Samaniego *et al.* 1997).

A different approach to reducing toxicity was to delete or inactivate the VP16 gene and therefore prevent transactivation of all of the IE genes. The protein cannot be deleted as it has an essential structural role, but it can be mutated in the C-terminal domain so that it cannot transactivate the IE genes but still retains its structural integrity (Ace *et al.* 1989). The resulting virus gives a 90% reduction in IE gene expression. This mutation can be complemented by adding hexamethylene bisacetamide (HMBA) to the growth media (McFarlane *et al.* 1992). This mutation has been combined with deletions of ICP0 and ICP4 to give a virus with no visible cytotoxicity to cells (Preston and Nicholl 1997). This virus was subsequently used to deliver long-term gene expression in the PNS (Marshall *et al.* 2000).

However, when the VP16 mutation is combined with IE gene deletions in a virus, complementation by HMBA is insufficient to give effective virus growth. To overcome this problem, cell lines expressing the equine herpesvirus1 homologue of VP16 (EHV1 gene 12) along with ICP4 and ICP27 were developed and found to successfully grow multiply disabled viruses in culture (Thomas *et al.* 1999b). This combination of deletions and mutation of VP16, along with a deletion in the ICP34.5 gene has allowed

development of a viral vector completely replication incompetent that can efficiently be grown in culture and widely express transgene in the brain for at least 1 month without causing damage (Lilley *et al.* 2001).

Persistent expression of transgenes in the brain has however not been achieved very effectively long-term, even with apparently non-toxic vectors. Latency promoters appear to have altered kinetics in the CNS to the PNS and seem to turn off over time. This is seen with the virus described above (Lilley *et al.* 2001) and also with other viruses such as a VP16-/ICP0-/ICP4- virus that expresses β-gal under the control of the LAT promoter region for up to 180 days in the brain, but this can only be detected by PCR after 4 weeks (Scarpini *et al.* 2001). One group claim that use of a multiply-disabled virus expressing transgenes under the control of the LAP2 promoter only, allows continued expression in the brain for at least 6 months, in biologically relevant quantities (Puskovic *et al.* 2004), although as LAP1 and not LAP2 has been shown to be active during latency, this is somewhat questionable.

The level of replication-competence required in a vector appears to differ for the PNS and CNS. Studies in the PNS show that a virus just deleted for ICP34.5 and with a mutated VP16 is non-toxic to dorsal root ganglia (DRG) when injected into the footpad (Coffin *et al.* 1996; Palmer *et al.* 2000). Further deletions of ICP4 and ICP27 reduced latent gene delivery in the PNS, suggesting that a certain amount of initial viral replication is advantageous in this case for latent transgene expression (Palmer *et al.* 2000). However, better transgene delivery in the brain is given by a virus additionally deleted for ICP4 and ICP27, as discussed above (Lilley *et al.* 2001).

Applications of optimised HSV1 vectors are potentially very wide reaching. Vectors can be used to deliver potentially therapeutic molecules to neurons *in vitro* and *in vivo,* which as well as a therapeutic tool is also a useful means of research into neurological diseases.

Examples of such applications are the delivery of Bcl-2 and GDNF to the rat substantia nigra, allowing 6-hydroxydopamine-induced degeneration of these neurons to be prevented (Natsume *et al.* 2001; Yamada *et al.* 1999). The opioid precursor preproenkephalin was delivered to mice spinal ganglia and demonstrated to have an anti-hyperalgesic effect (Wilson *et al.* 1999). Also, a disabled vector expressing NGF has been produced and shown to protect rat DRG neurons in an *in vitro* model of oxidative stress (Goins *et al.* 1999). Parkinson's disease is a good contender for HSV1 mediated gene therapy and delivering tyrosine hydroxylase (TH) to produce dopamine by HSV1 has been shown (During *et al.* 1994), as has using a combination of the delivery of TH and aromatic acid decarboxylase (Sun *et al.* 2003). These are just a few examples of a wide-range of uses for which HSV1 vectors have been suggested.

1.2 AN OVERVIEW OF TRANSCRIPTIONAL REGULATION

1.2.1 Promoters

In eukaryotic transcription, the promoter is the site where the RNA pol II binds to the DNA to initiate transcription. Promoters of genes that synthesise mRNA are typically located immediately upstream of the initiation site. Some, but not all of these promoters contain the consensus sequences TATAAA (known as the TATA box), where the RNA polymerase binds. In humans approximately 32% of promoters possess a TATA box (Suzuki *et al.* 2001). The TATA box is usually about 30bp upstream of the first base to be transcribed.

Initiator (Inr) elements are found in both TATA-containing and TATA-less promoters. This is a consensus sequence surrounding the start site for transcription.

Downstream core promoter elements (DPEs) are most commonly found in TATA-less promoters and are located 28 to 32 bases downstream of the Inr site. The core promoter elements are illustrated in figure 1-7.

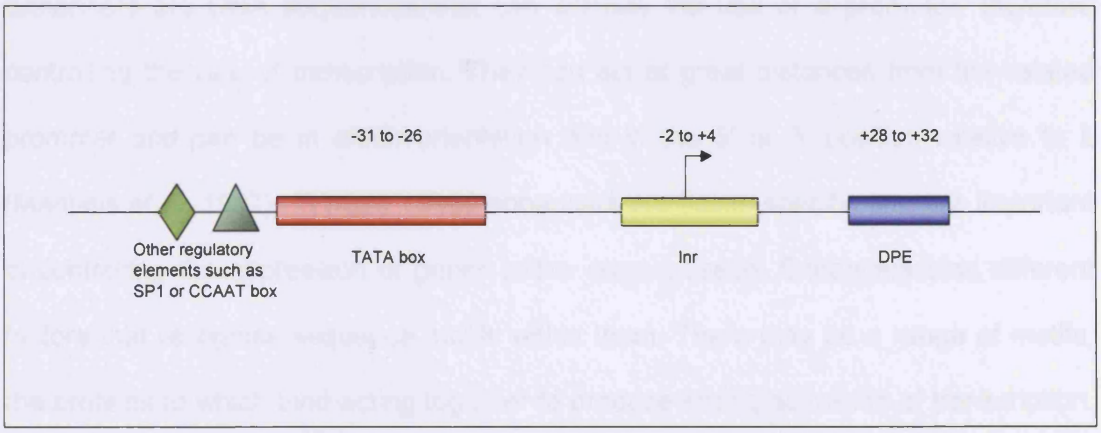


Figure 1-7 Core promoter elements

Numbering refers to +1 as being the starting point of transcription within the Inr element.

The RNA polymerase II cannot bind to naked DNA, but requires TFs to bind efficiently. At least 6 basal TFs are required, known as TFIIA, B, D, E, F and H (Buratowski *et al.* 1989; Sopta *et al.* 1989). TFIIB and D are the first two factors to bind to the promoter and thus have a critical role in the recognition of core promoter motifs. This then allows recruitment of RNA pol II, either independently of the other TFs, or along with them in a holoenzyme form. This holoenzyme contains other protein components that appear to be involved in opening the chromatin structure (see later) and allowing in further TFs.

TFIID binds the TATA box where present, or in the absence, binds to another DNA binding protein present which in turn recognises the Inr element. In this way the transcription initiation complex (TIC) is brought to the correct location for transcription to occur.

Control of transcription occurs by one way or another altering the number of RNA pol II molecules moving along the DNA i.e. by activation or repression.

1.2.2 Enhancers

Enhancers are DNA sequences that can activate the use of a promoter, therefore controlling the rate of transcription. They can act at great distances from the related promoter and can be in either orientation and in the 5' or 3' position relative to it (Maniatis *et al.* 1987). In many cases enhancers are tissue-specific and are important in controlling the expression of genes in the correct tissue. Enhancers bind different factors that recognise sequence motifs within them. There may be a range of motifs, the proteins to which bind acting together to produce strong activation of transcription. The complex of different proteins bound to an enhancer is called an enhanceosome.

It is thought that enhancers function by causing a change in the chromatin structure leading to nucleosomal displacement (see later) and/or by direct interaction with the proteins of the transcriptional apparatus. Thus the enhanceosome complex recruits both chromatin modifiers and the RNA polymerase II holoenzyme to direct enhanced transcription (Giese *et al.* 1995; Grosschedl 1995; Thanos and Maniatis 1995). When direct interaction of the enhancer with the transcriptional apparatus occurs and the enhancer is a long distance from the promoter, then it is thought that 'looping out' of the intervening DNA probably occurs.

Silencers are the opposite of enhancers in that they repress rather than activate transcription. Apart from that, the mechanisms of action are probably similar, allowing inhibitory proteins to bind to the transcriptional apparatus, or causing a less permissive chromatin structure to form (Brand *et al.* 1985).

As enhancers and silencers are often separated from the promoter of intent by other promoters that are not affected, insulator elements provide a means by which inappropriate transcription or repression of such is prevented. Insulator elements are sequences that provide a 'block' to the enhancer or silencer activity, the way in which they function depending on the mechanism of action of the enhancer/silencer. They bind a protein that either prevents the enhancer being brought into contact with the promoter, or that prevents the opening or closing of the local chromatin structure.

1.2.3 Transcriptional regulation by epigenetic mechanisms

1.2.3.1 Chromatin structure

Eukaryotic DNA exists in a compacted form, associated with proteins in a form known as chromatin. The associated proteins are called histones, which are very rich in the amino acids lysine (K) and arginine (R), which causes the histone to be positively

charged and thus tightly bind the negatively charged DNA, by electrostatic interactions.

The basic repeat unit of chromatin is the nucleosome, which consists of a histone octamer made up of 2 molecules each of the four core histones H2A, H2B, H3 and H4. Approximately 146bp of DNA is wound around the core, by almost 2 full turns. One histone H1 molecule binds to the outside of the nucleosome and allows tighter winding of the DNA (Kornberg 1974; Olins and Olins 1974; Van Holde *et al.* 1974).

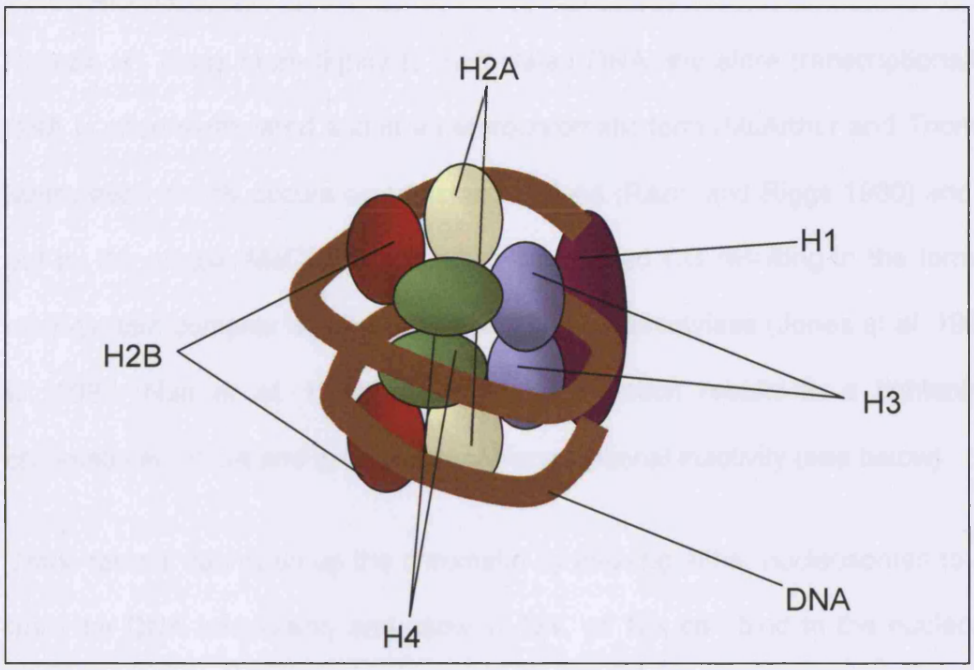


Figure 1-8 Schematic diagram of a nucleosome

The histone proteins H2A, H2B, H3 and H4 exist as dimers, making up the core of the histone, with 146bp of DNA wrapped around them. A single histone H1 helps to bind the nucleosome.

The nucleosomes are joined together by small stretches of linker DNA. DNA that is in a nucleosomal form can be digested by micrococcal nuclease (MNase) between the nucleosomes to give a ladder of characteristically sized fragments, relating to the number of nucleosomes per fragment (the mono-nucleosomal band is 146bp) (Lacy and Axel 1975).

Tightly bound chromatin is known as heterochromatin and is largely inaccessible to the transcriptional machinery. Actively transcribed genes, or those with the potential to be transcribed are also found arranged on nucleosomes, but the chromatin is in a more open structure known as euchromatin, which allows the transcriptional machinery to access the DNA. Stretches of euchromatin can be identified by digestion with DNase1,

which will only digest DNA not associated with nucleosomes and that is accessible to the enzyme (Weintraub and Groudine 1976).

Histone H1 binds more tightly to methylated DNA, therefore transcriptionally inactive DNA is often methylated and in a heterochromatic form (McArthur and Thomas 1996). Methylation mainly occurs on cytosine residues (Razin and Riggs 1980) and is carried out by the protein MeCP2 which binds methylated CG resulting in the formation of a multi-protein complex which includes a histone deacetylase (Jones *et al.* 1998; Nan *et al.* 1997; Nan *et al.* 1998). Histone deacetylation results in a tightening of the chromatin structure and is a marker of transcriptional inactivity (see below).

Trans-factors can open up the chromatin by causing either nucleosomes to dissociate from the DNA temporarily and allow in TFs, or TFs can bind to the nucleosome and disrupt the structure (Adams and Workman 1993). Once nucleosomes in the promoter region of a gene are disrupted then other TFs can bind. DNase 1 sites are often found at enhancers, suggesting that enhancers allow binding of factors that then alter the local chromatin structure and facilitate transcription from the related promoter (Zaret and Yamamoto 1984). There are two classes of chromatin modifying factors: histone modifiers and ATP-dependent nucleosome remodelling complexes. These are both described below.

1.2.3.2 Histone modification in transcriptional control

The core histone tails protrude from the nucleosome and are susceptible to a variety of covalent modifications including methylation, phosphorylation, ubiquitination and acetylation; the latter being the most common. The modifications alter the structure of the chromatin in different ways, either by causing a direct tightening or loosening by allowing the histones to more tightly interact, or by indirectly allowing facilitation of other modifications to occur.

Histones H3 and H4 can be methylated on R and K residues and although this has been known for a long time, the relevance was not understood (Murray 1964). The function was unknown due to the lack of knowledge about methylating enzymes. Thus, when methyltransferases were discovered this provided evidence for the involvement of methylation in transcriptional regulation (Chen *et al.* 1999; Rea *et al.* 2000; Schek and Bachenheimer 1985). Methylation of histones is associated with transcriptional inactivation.

Phosphorylation of the core histones can also occur; although it appears to be an indirect regulator of transcriptional activity. For example phosphorylation of S10 on H3 inhibits methylation of K9 on H3 and promotes acetylation of K14 on H3, by creating a more favourable substrate for histone acetyl-transferases (HATs). This then leads to transcriptional activation (Cheung *et al.* 2000; Lo *et al.* 2000; Sassone-Corsi *et al.* 1999).

Histones H2A and perhaps H2B are able to be ubiquitinated. This modification is associated with increased transcriptional activity and is prevalent at the 5' end of transcriptionally active genes (Varshavsky *et al.* 1982).

Acetylation of histones was first linked to transcriptional activation in the 1960s (Allfrey *et al.* 1964). Histones with acetylated N-terminal histone tails bind DNA and other histones of adjacent nucleosomes with reduced affinity and thus allow a loosening of the nucleosomal and chromatin structure (Hong *et al.* 1993; Puig *et al.* 1998). When the first nuclear acetylase GCN5 was discovered (Brownell *et al.* 1996) and the first deacetylase, HDAC1 (Taunton *et al.* 1996), the importance of histone acetylation as a method of transcriptional control was underlined. It was then found that other activators of transcription have HAT activity such as p300/CBP and the $TAF_{II}250$ subunit of TFIID (Mizzen *et al.* 1996; Ogryzko *et al.* 1996; Yang *et al.* 1996).

HATs function by transferring an acetyl group from acetyl-coenzyme A to the ε-amino acid group of lysines on H3 and H4, reducing the net positive charge and therefore the interaction with the DNA and other histones, such that the nucleosome structure is loosened (Allfrey *et al.* 1964). Acetylation and deacetylation occur in synergy to achieve appropriate levels of transcription.

1.2.3.3 Nucleosome remodelling complexes

The second class of chromatin modifier is the ATP-dependant nucleosome-remodelling complexes such as the SWI/SNF family. These work by allowing an exchange between condensed and more accessible nucleosome configuration, either through histone octamer transfer and/or nucleosome sliding (Hamiche *et al.* 1999; Schnitzler *et al.* 1998; Whitehouse *et al.* 1999). The SWI/SNF complexes are thought to be recruited to promoters via interactions with DNA binding proteins, as they do not recognise specific sequences (Vignali *et al.* 2000). These could be either transcriptional activators as in the case of the glucocorticoid receptor that has been shown to recruit SWI/SNF to the responsive promoter (Deroo and Archer 2001), or transcriptional repressors such as UME6 (Goldmark *et al.* 2000).

1.2.3.4 Interaction of chromatin remodellers and histone modifiers

The covalent modifiers of histones and the ATP-dependent chromatin remodellers are likely to work together to modulate transcriptional activation and repression. Studies on the yeast HO promoter showed that the SWI/SNF complex and then a HAT binds to it prior to transcription and that the SWI/SNF complex is required if acetylation is to occur (Cosma *et al.* 1999; Krebs *et al.* 1999). It has been shown that the inter-dependence is more likely to affect deacetylation rates (Imbalzano *et al.* 1994; Logie *et al.* 1999; Tong *et al.* 1998). However, this is not a strict rule; the two can usually function independently.

One model of co-operation proposed is that the SWI/SNF complex creates a 'fluid' chromatin environment which is then locked into place by covalent modifications of histone tails (Kingston and Narlikar 1999). It is hypothesised that alteration of the chromatin by one method may make it easier for the other to work e.g. by increasing accessibility to the DNA or histones. It does appear that there is no strict order in which these different actions of chromatin remodelling and modification occur and that they are likely to function synergistically to regulate transcription.

1.2.3.5 Insulators and barriers

As a genome has a combination of silenced and active genes, these are organised into chromatin domains that are maintained independently to their surroundings through the establishment of chromatin boundaries.

As mentioned above, (section 1.2.2), insulator elements bind proteins that can provide a block to regulatory elements such that their action is not imparted onto inappropriate genes. Another function of insulators is to prevent the self-propagating spread of chromatin condensation onto neighbouring gene *loci* thus affecting gene expression (Elgin and Grewal 2003). There are two types of insulator function – one that blocks the effect of enhancer activity and one that creates barriers to compartmentalise the different chromatin conformations.

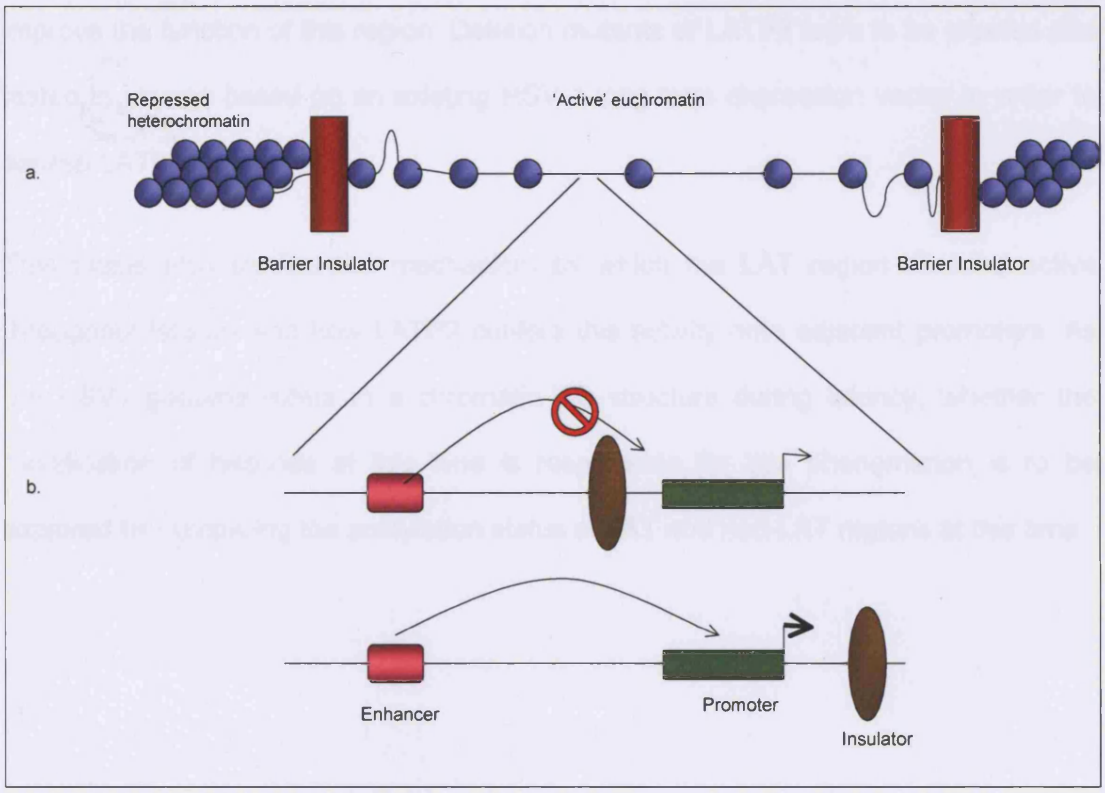
Although the two functions are separate, they can exist within the same factor. For example, the HS4 element contains both functions. The insulator function requires the protein CCCTC binding factor (CTCF) to bind (Bell *et al.* 1999) as do a number of different insulator elements. The barrier function of the HS4 element does not however require CTCF to function (Recillas-Targa *et al.* 2002).

A sharp transition between the hypoacetylated inactive region and the hyperacetylated active chromatin domain is seen at the locus of a barrier insulator (Hebbes *et al.* 1994) and it has been shown that the maintenance of transcriptional activity within the

flanking barrier elements of a region is dependent upon continued acetylation which in turn is dependent upon the presence of the barrier elements. This led to the hypothesis that insulators prevent histone deacetylation within the active region (Pikaart *et al.* 1998). Evidence to back up this theory came when the HS4 insulator was found to be hyperacetylated, suggesting that it attracts a high-level of histone acetylases. Furthermore, maintenance of acetylation was essential for HS4 to prevent the silencing of transgenes placed adjacent to it (Litt *et al.* 2001a; Litt *et al.* 2001b; Mutskov *et al.* 2002).

It has been found that the active chromatin region must be flanked on both ends by insulator barriers to protect it from silencing. A barrier at one end only has no effect (Pikaart *et al.* 1998).

The two different functions of insulators can be exemplified by the fact that within an active chromatin domain, flanked by barriers, insulators of enhancer function can also exist. Figure 1-9 provides an illustration of how this can occur.



Adapted from (Burgess-Beusse *et al.* 2002)

Figure 1-9 Two types of insulator function

a. Barrier function allows distinct compartments of active euchromatin without the encroachment of adjacent condensed chromatin.
b. Enhancer-blocking function prevents activation of inappropriate promoters when placed between enhancer and promoter.

1.3 THESIS AIMS

As shown previously, the LATP2 region of HSV1 has properties that appear to allow continued expression of transgenes from LAT or non-LAT promoters, which would otherwise not be active during viral latency. This has great potential in the design of HSV1 vectors for gene therapy, where continued expression of therapeutic genes is often required. To optimise the long-term expression capabilities of the vectors, it would be advantageous to understand how the LATP2 region confers this ability onto other promoters.

This thesis therefore aims to identify which regions of LATP2 are responsible for the long-term expression properties and which, if any, could be dispensed with in order to

improve the function of this region. Deletion mutants of LATP2 were to be created and tested in viruses based on an existing HSV-1 long-term expression vector in order to assess LATP2 activity.

This thesis also studies the mechanism by which the LAT region remains active throughout latency and how LATP2 confers this activity onto adjacent promoters. As the HSV1 genome exists in a chromatin-like structure during latency, whether the modification of histones at this time is responsible for this phenomenon is to be explored by comparing the acetylation status of LAT and non-LAT regions at this time.

CHAPTER 2:

MATERIALS AND METHODS

2.1 LABORATORY REAGENTS

Unless otherwise stated, all chemicals were from Merck Ltd. (Poole, Dorset, UK), Roche Biochemicals (Lewes, East Sussex, UK) or Sigma Chemical Company Ltd. (Poole, Dorset, UK) and were of analytical grade. General disposable plasticware was purchased from Sterilin (Stone, Staffordshire, UK) or Greiner (Stonehouse, Gloucester, UK).

Additional laboratory materials and reagents were supplied from one of the following:

Insight Biotechnology Ltd. (London, UK); Nunc (Roskilde, Denmark); Amersham International plc. (Little Chalfont, UK); Qiagen (Chatsworth, USA); Gibco-BRL Life Technologies Ltd. (Paisley, Renfrewshire, UK); Bio-Rad (Hemel Hempstead, UK); Promega Corporation (Madison, Wisconsin, USA); Whatman International Ltd. (Maidstone, Kent, UK); Pharmacia Biotechnology Ltd. (St Albans, UK); Millipore Ltd. (Watford, UK); Stratagene Ltd. (Cambridge, UK); Marligen Bioscience Inc. (USA); New England Biolabs Inc. (Hitchin, Hertfordshire, UK); Upstate Biotechnology (Buckinghamshire, UK).

All oligonucleotide primers were constructed by Invitrogen Custom Primers (Paisley, Renfrewshire, UK).

2.1.1 Standard buffers and solutions

PBS: NaCl (137mM), KCl (2.7mM), $Na_2HPO_4.7H_2O$ (4.3mM), KH_2PO_4 (1.4mM)

TE: Tris-HCl (10mM), EDTA (1mM), pH8.0

TAE: Tris base (400mM), sodium acetate (200mM), EDTA (20mM), pH8.3

Luria Bertani (LB) media: 1% (w/v) Bacto®-tryptone
1% (w/v) NaCl
0.5% Bacto®-yeast extract

2.2 MOLECULAR BIOLOGY

2.2.1 Bacterial strains and growth conditions

The XL-1 Blue (Stratagene Ltd, Cambridge, UK) strain of *E.coli* was used for all plasmid cloning.

XL1-Blue recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F'proAB $LacI^q$ ZΔM15, TN 10 $(Tet)^r$]

2.2.1.1 Propagation of bacteria

XL1-Blue (XL1-B) cells were grown in LB media containing either no antibiotic or 100µg/mL ampicillin overnight in a Gallenkamp orbital shaker at 200rpm/37°C. LB media was autoclaved at 120°C for 20 mins at 10lb/square inch. Stocks of ampicillin were made at 100mg/mL in 50% (v/v) ethanol and stored at -20°C for up to 3 months. Colonies of XL1-B were isolated by growing on LB plates containing 2% Bacto®-agar.

2.2.1.2 Long-term storage of bacteria

An overnight culture of the XL1-B cells was diluted 1:10 into fresh LB media and incubated with shaking at 200 rpm at 37°C for 1 hour. An equal volume of 100% glycerol was then added and the two solutions were thoroughly mixed. The glycerol/bacteria solution was aliquoted into cryo-vials and frozen at –80°C.

2.2.1.3 Preparation of competent XL1-blue cells

Competent XL1-B cells were prepared using a standard calcium chloride technique (Sambrook *et al.* 1987). A 5mL starter culture (containing no antibiotic) of XL1-B was grown overnight at 200rpm/37°C, which was then used to inoculate 100mL of LB (containing no antibiotic) and the culture grown to an OD_{580} of 0.4-0.55 units. The bacteria were then pelleted in a Jouan CR422 centrifuge at 2000rpm for 10 mins at 4°C and any excess LB removed. The cells were resuspended in 10mL ice-cold

100mM $CaCl_2$ and incubated on ice for 1 hour. The cells were then pelleted as before, resuspended in 4mL ice-cold $CaCl_2$ and then incubated on ice until required.

2.2.1.4 Transformation of XL1-blue cells

200µL competent XL1-B cells were transformed by the addition of DNA and subsequent incubation on ice for 20 mins. The cells were then heat-shocked for 90 secs. at 42°C. The cells were then incubated on ice for a further 2 mins. 800µL LB was then added to the cells and then incubated in an orbital shaker for 1 hour at 37°C and 200rpm. The cells were then pelleted and resuspended in 100µL of LB and plated onto LB-agar plates containing 100µg/mL of ampicillin. If detection of β-Galactosidase was required, plates containing 50µL of 20mg/mL stock of 4-Cl, 5-bromo, 3-indolyl-β-galactosidase (X-Gal, Insight Biotechnology Ltd., UK) dissolved in DMF were used.

2.2.2 DNA extraction and purification

2.2.2.1 Small-scale plasmid DNA extraction and purification

The "mini-prep" extraction method used is based on an alkaline lysis method (Birnboim and Doly 1979). Single colonies of XL1-B cells were used to inoculate 5mL LB containing 100µg/mL ampicillin and were incubated in an orbital shaker overnight at 200rpm/37°C. The cells from 1.5mL of culture were then pelleted by centrifugation in an Eppendorf 5417C microfuge at 9000rpm for 1 min at room temperature. The cells were then resuspended in 100µL resuspension buffer (50mM Tris-HCL pH7.5, 10mM EDTA pH 8, 100µg/mL RNase-A). Cells were lysed by addition of 200µL lysis buffer (200mM NaOH, 1% (v/v) Triton X-100) and then 150µL of neutralisation buffer (3M NaOAc, pH5.5) was added. The cell lysate was centrifuged in an Eppendorf 5417C microfuge for 3 mins at 13000rpm at room temperature and the pelleted precipitate removed with a hypodermic needle bent at the tip. 500µL of isopropanol was added to

the remaining supernatant, mixed and then centrifuged for 5 mins at 13000rpm at room temperature. The supernatant was removed and the DNA pellet washed with 500µL 70% (v/v) ethanol. The pellet was air dried and then resuspended in 50µL of double-distilled water (ddH_20). Plasmid 'mini-prep' DNA was stored at -20°C.

2.2.2.2 Large-scale plasmid DNA extraction and purification

Large-scale plasmid DNA extractions were performed to produce high grade DNA for cloning, isolation of DNA fragments and transfections (section 2.3.4.1). 100µL of an overnight culture of transformed XL1-B cells was used to inoculate 400mL LB containing 100µg/mL ampicillin and incubated in an orbital shaker overnight at 200rpm/37°C. 100mL of the culture was centrifuged at 3500rpm for 10mins at room temperature. Plasmid DNA was then extracted using the Concert Midi-Prep kit (Marligen, USA) as per the manufacturer's instructions. A typical yield of 'midi-prep' DNA was 200µg, which was resuspended in 200µL ddH_20. Exact quantification of the DNA in each preparation was done by measuring the absorbance of the DNA at 260nm in a UV spectrophotometer. The concentration of DNA was calculated using the Beer-Lambert equation such that concentration (µg/mL) = AU x 50x dilution factor.

2.2.3 Cloning

2.2.3.1 Restriction digests

Restriction digests were performed on plasmid DNA for clonal analysis. Digests were carried out in a total volume of 20µL, containing either 5µL of 'mini-prep' DNA (section 2.2.2.1) or 1µL of 'midi-prep' DNA (section 2.2.2.2). 10 units of each restriction enzyme were added in the appropriate buffer. The volume was made up to 20µL with ddH_20. Digests were incubated from 1 hr – overnight at the appropriate temperature for the enzyme being used. Digestion products were then run on a 0.8 - 2% agarose gel

(dependent on expected band sizes) (section 2.2.3.5) and visualised on a UV transilluminator.

2.2.3.2 Isolation of DNA fragments

Restriction digests required for isolation of DNA fragments for cloning were carried out in a total volume of 20µL containing ~5µg 'midi-prep' DNA, 10 units of each enzyme and the appropriate buffer. The volume was made up to 20µL with ddH_20. Digests were incubated at the appropriate temperature for 1 hr – overnight. Digestion products were then run on a 1% low-melting-point agarose gel and visualised on a UV transilluminator. Required bands were carefully excised using a scalpel blade. DNA was then extracted from the agarose using the GFX™ PCR and Gel Band Purification Kit (Amersham, UK) as per the manufacturer's instructions. The DNA was eluted in a final volume of 20µL ddH_20.

2.2.3.3 Blunt-end reactions

When there were no compatible restriction sites for cloning, sticky-ends were blunted by filling in their 3' overhangs using T4 DNA polymerase (Promega, USA). After restriction digest, 1µL of a 25mM stock dNTPs (dATP, dCTP, dGTP, dTTP) and 15 units of T4 DNA polymerase were added. The reaction was incubated for 30 mins at 37°C. If subsequent restriction digests were to be performed, the reaction was heat-inactivated at 80°C for 30 mins and then cooled on ice prior to the addition of further restriction enzymes.

2.2.3.4 Phosphatase treatment

To prevent possible religation of vector ends, the vector DNA was dephosphorylated using shrimp alkaline phosphatase (SAP). Vector DNA was extracted using the GFX™ PCR and Gel Band Purification Kit (Amersham, UK) according to manufacturer's instructions. DNA was resuspended in a total volume of 50µl ddH_2O containing 1x phosphatase buffer and 5 units SAP. The reaction was incubated for 45 mins at 37°C,

then 10 mins at 65°C to inactivate the SAP. DNA was again extracted as before and resuspended in 10µl ddH_2O.

2.2.3.5 Agarose gel electrophoresis

0.5-1.5% (w/v) agarose gels were made using 1x TAE (0.4 M Tris base, 0.2 M sodium acetate, 20 mM EDTA pH8.3). Ethidium bromide was added to a final concentration of 0.5µg/ml. Approximately 0.1 volume of 10x loading buffer (1x TAE, 50% v/v glycerol, 0.025% bromophenol blue) was added to DNA samples prior to loading. 1 kilobase (kb) ladder DNA marker was used as a size marker. DNA was electrophoresed at 80-120mA until the fragments were well separated. Bands were visualised on a UV transilluminator and photographed with Polaroid film.

2.2.3.6 Phenol extraction of DNA

When DNA restriction digestions needed to be purified, a phenol extraction was performed by the standard technique (Sambrook *et al.* 1987; Sambrook and Russell 2001). This was necessary if a digestion required two enzymes and a compatible buffer could not be found, or when linearised DNA was used in transfections (section 2.3.4.1). Digestions were made up to a total volume of 400µL with ddH_20. An equal volume of phenol/chloroform/IAA (24:24:1) was added and the mixture vortexed thoroughly before centrifugation at 12000rpm for 2 mins in an Eppendorf 5417C microfuge. The aqueous layer was removed and an equal volume of chloroform/IAA (24:1) was added. The mixture was vortexed thoroughly and pulse-centrifuged. The aqueous layer was removed and to this was added 40µL (0.1 x volume) of 3M NaOAc (pH5.5) and 880µL (2 x volume) of ice-cold ethanol. The mixture was vortexed and then left at -70°C for 10 mins, before centrifugation for 15 mins at 13000rpm, 4°C in an Eppendorf 5417R microfuge to pellet the DNA. The supernatant was removed and the DNA washed in 70% ethanol before being left to dry. The extracted DNA was then resuspended in 10µL ddH_20.

2.2.3.7 DNA ligations

Ligations were performed in thin walled 0.5mL reaction tubes in a total volume of 20µL. Reactions contained 1-2µL of purified vector, 7-10µL of purified insert, 1 x ligase buffer, 1-3 units of T4 DNA ligase (Promega, USA) and made up to volume with ddH_20. Reactions were left either at room temperature for 2 hrs, or in the case of blunt-end ligations in a thermocycler. The thermocycler was run at 16°C for 1 min followed by 37°C for 1 min for 30 cycles and a final incubation at 22°C for 30 mins. In each case the ligation reaction was transformed into 200µL of competent XL1-B cells as described (section 2.2.1.4).

2.2.3.8 Colony characterisation using a radiolabelled probe

In the absence of X-Gal mediated selection, bacterial colonies could be screened for the incorporation of an inserted DNA fragment using a radiolabelled probe. This method was of particular use following blunt-end ligations where the insertion efficiency was usually low.

2.2.3.8.1 Colony transfer

Bacterial colonies were directly transferred onto Hybond-N+ nitrocellulose membranes (Amersham, UK) by placing membranes onto the LB-agar plates for 2 mins. Holes were punched in the membrane in an asymmetric pattern using a sterile needle. The position of the holes was marked on the Petri dish with a pen for the purpose of orientation at a later stage. The bacterial cells were lysed and the DNA denatured by immersing the nitrocellulose membranes in denaturing solution (1.5M NaCL, 0.5M NaOH) for 2 mins. The filters were then immersed in neutralisation solution (2M NaCL, 1M Tris pH5.5) for 2 mins and washed in 2x SSC/0.1% SDS for 2 mins. The membranes were washed twice in 2 x SSC for 2 mins. Placing the membranes colony side up, they were cross-linked twice in a UV stratalinker (Stratagene, Netherlands).

2.2.3.8.2 Radiolabelling DNA probes

Fragments of DNA were radiolabelled with α- [^{32}P]-dCTP. DNA was digested (section 2.3.1.2) and run on a 1% agarose gel (section 2.2.3.5). The required DNA was then excised from the gel and purified with the GFX™ PCR and Gel Band Purification Kit (Amersham, UK) according to manufacturer's instructions. The DNA was eluted in 20µL TE buffer. To label the probe the 'Ready-To-Go™ DNA labelling beads' (Amersham, UK) were used as per the manufacturers instructions, using all of the eluted probe DNA in the reaction. The radiolabelled probe was then heated for 5 mins at 100°C and snap-cooled on ice for 2 mins before adding to the hybridisation solution.

2.2.3.8.3 Hybridisation

The nylon membranes were pre-hybridised for between 2-5 hrs at 65°C with 30mL of pre-hybridisation solution (6x SSC, 5x Denhardt's reagent [Denhardt's Reagent (100x): 2% (w/v) bovine serum albumin, 2% Ficoll® (type 400), 2% (w/v) polyvinylpyrrolidone in dd$H_2$0], 0.5% (w/v) SDS in dd$H_2$0 containing 100µg/mL denatured herring sperm DNA). The volume of the pre-hybridisation solution was reduced to 5mL prior to adding a denatured probe. The probe for the appropriate DNA fragment was hybridised to the membranes overnight at 65°C. The membranes were washed twice for 10 mins in 2xSSC/0.1% (w/v) SDS. The membranes were then wrapped in cling-film and exposed to X-ray film at -70°C. If necessary the membranes were washed further in 0.5xSSC/0.1% (w/v) SDS and then 0.1xSSC/0.1% (w/v) SDS.

2.2.4 Polymerase chain reaction (PCR)

The specific PCR amplification of a target DNA sequence was achieved using methodology essentially as described by Saiki *et al* (Saiki *et al.* 1985). Specific conditions are shown below and elsewhere in the text.

2.2.4.1 Standard PCR reaction.

The target DNA was added to the PCR reaction, which contained: 10x PFU or Taq buffer (Promega, USA), 40mM dNTPs, 50-100ng of each primer (Invitrogen, UK), 2.5units of Taq or PFU polymerase (Promega) and the final volume was made up to 50μl. To prevent evaporation of the reaction of the mix during thermal cycling the reaction was overlaid with 50μl of mineral oil. The PCR reactions were then put through different thermal cycles according to the primers used.

2.2.4.2 PCR of viral DNA

Due to the GC rich nature of HSV DNA, when it was used in a PCR then the GC-Rich PCR System (Roche, East Sussex, UK) was employed. This was used according to the manufacturer's instructions in varying thermal cycles according to the primers used.

2.2.5 Preparation of cDNA

All solutions used in this process were treated with diethylpyrocarbonate (DEPC). RNA was prepared using Trizol reagent (Invitrogen, UK) according to the manufacturer's instructions. RNA was dissolved in 25μl of DEPC H_2O.

First-strand cDNA synthesis was performed using Superscript II Reverse Transcriptase (Invitrogen, UK). RNA (1-5ng) was added to a tube with 150ng random primers (Invitrogen, UK), 40mM dNTP and was made up to 20μl with DEPC H_2O. This mixture was then heated to 65°C for 5 mins and chilled on ice for 5 mins. Then 4μl 5x first-strand buffer (100mM HEPES pH 6.9, 2mM EDTA), 2μl 0.1M DTT and 40U

RNasin (Promega, US) were added and incubated at 42°C for 2 mins. 1μl of Reverse Transcriptase (Superscript II, Invitrogen, UK) was added and the reaction was incubated at 25°C for 10 mins, followed by 42°C for a further 50 mins. Finally the Reverse Transcriptase was denatured by incubation at 70°C for 15 mins. 10% of the cDNA was then added to a standard PCR reaction as described in section 2.2.4.1.

2.2.6 DNA sequencing

Where sequencing of DNA was necessary (e.g. after PCR) then this was carried out by the sequencing service, department of Biochemistry, University of Cambridge.

2.2.7 Viral DNA preparation

A well of a 6 well plate of virally infected cells at complete cytopathic effect (CPE) was harvested by adding 1ml of DNAzol (Helena Biosciences, Sunderland, UK). The DNA was precipitated by adding 0.5x volume of absolute ethanol and washed twice in 75% ethanol. Ethanol was removed and the DNA pellet dried for 5mins at room temperature (RT). The DNA was resuspended in 200μl of 8mM NaOH and incubated overnight at 4°C on a rotating wheel. For neutralization, 23μl of 0.1M HEPES were added and the DNA stored at -20°C.

2.2.8 Southern blotting

Southern blots (Southern 1975) were performed on viral DNA to determine that correct homologous recombination had occurred when producing recombinant HSV viruses.

2.2.8.1 DNA preparation

A varying amount of DNA was digested with the appropriate enzymes and buffers in a total of 50μL overnight. The digested reactions were then run on a 1% TAE agarose gel until DNA fragments were well separated. The DNA was visualised on a UV transilluminator and photographed with a fluorescent ruler.

2.2.8.2 DNA transfer

The gel was left on the transilluminator for 2 mins and for further denaturation was then placed in denaturing solution (1.5M NaCL, 0.5M NaOH) for 45 mins. After rinsing briefly in H_20, the gel was transferred to neutralising solution (2M NaCL, 1M Tris pH5.5) for at least 30 mins. The gel was then placed upside-down on a support covered in a layer of 3MM Whatman paper (Whatman, Maidstone, UK) which was used as a wick placed in a reservoir of 20x SSC (150mM NaCL, 15mM sodium citrate pH8.0). A piece of Hybond-N+ nylon membrane (Amersham, UK) cut to the same size as the gel was pre-soaked in the neutralising solution and then carefully placed on the gel ensuring that there were no air bubbles present. 10 pieces of 3MM Whatman paper, pre-soaked in 20x SSC were placed on top of the nylon membrane and a stack of dry paper towels was placed on top with a weight. The DNA was then transferred by capillary action from the gel to the nylon membrane overnight. The membrane was removed and washed in 6x SSC and then cross-linked twice using a UV stratalinker. The membrane was then air dried for 30 mins. The fixed membrane could then be stored at 4°C if required, prior to hybridisation.

2.2.8.3 Membrane analysis

Southern blot membrane analysis was performed using a radiolabelled probe designed to hybridise to the region of interest. The probe was labelled as previously described (section 2.2.3.8.2) and hybridisation was carried out as in section 2.2.3.8.3.

2.3 TISSUE CULTURE

Tissue culture preparations were carried out under sterile conditions in a class II safety cabinet (Heraeus). Viral preparations were carried out under Health and Safety Category 2 Conditions. Tissue culture plastic ware was obtained from Nunc, and media and supplements were supplied by Gibco-BRL.

2.3.1 Mammalian cell lines

Dulbecco's modified Eagle Medium (DMEM) supplemented with 10% foetal calf serum (FCS), 100 units/mL penicillin/streptomycin, 2mM L-glutamine and 5% (v/v) Tryptose Phosphate Broth is referred to as full growth media (FGM) and was used for culture of all cell lines unless specified. DMEM supplemented with 100-units/mL penicillin/streptomycin is referred to as serum free media (SFM).

2.3.1.1 Baby hamster kidney cells

Baby Hamster Kidney Cells (BHKs) (MacPherson and Stoker 1962) – clone 21 were obtained from ECACC (number 85011433). Cells were passaged by washing in Hanks Balanced Salt Solution (HBSS) at room temperature and then incubated with a minimal volume of 10% (v/v) trypsin in versene at 37°C/5% CO_2. An appropriate volume of FGM was added to neutralise the trypsin/versene and the cells were then divided accordingly into fresh FGM and cultured at 37°C/5% CO_2.

2.3.1.2 27/12/M:4 cells

27/12/M:4 Cells (MAM49) are a stable cell line derived from BHK cells and have previously been described (Thomas *et al.* 1999b). The cells complement deletions or mutations in HSV-1 genes ICP4, ICP27 and VP16. Cells were cultured in FGM containing 800µg/mL of Neomycin and 750µg/mL of Zeocin (Cayla, France). Cells

were passaged as for BHK cells (section 2.3.1.1). When MAM49 cells were grown for viral infection antibiotics were omitted.

2.3.1.3 ND7 cells

ND7 cells were created by fusion of mouse neuroblastoma cells (N18Tg2) with a rat post-mitotic neonatal dorsal root ganglion neuron (Wood *et al.* 1990). Cells were cultured in Leibovitz L15 media supplemented with 10% FCS, 100 units/mL penicillin/streptomycin, 0.35% (w/v) glucose, 2mM L-glutamine and 0.375% (w/v) sodium bicarbonate. Cells were passaged by removal of media and addition of 10mL fresh L15 supplemented media followed by gentle agitation of the flask to dislodge the cells. The suspended cells were then divided as required into fresh L15 supplemented media.

2.3.1.4 Vero cells

Vero cells are an African green monkey kidney cell line (ATCC number CCL81). Cells were cultured in FGM and passaged as for BHK cells (section 2.3.1.1).

2.3.1.5 HT1080 cells

HT1080 cells are a human fibrosarcoma cell line (ATCC number CL121). Cells were cultured in FGM and passaged as for BHK cells (section 2.3.1.1).

2.3.2 Cell line storage

Cells were trypsinised (section 2.3.1.1) and centrifuged at 1500rpm for 10 mins to pellet them. The pellet was then resuspended in freezing media A (DMEM supplemented with 20% FCS) followed by addition of an equal volume of freezing media B (DMEM supplemented with 40% FCS and 16% dimethylsulfoxide [DMSO]). Cells were aliquoted into 1.5mL cryovials and were maintained at –80°C for 24 hours prior to storage in liquid nitrogen.

When required, frozen cell stocks were rapidly thawed at 37°C and transferred immediately to 10mL of fresh FGM and pelleted by centrifugation at 1500rpm for 10 mins in a Jouan CR422 centrifuge. The cell pellet was then resuspended in 6mL fresh FGM in a 25cm^2 flask and incubated at 37°C/5% CO_2.

2.3.3 Primary cultures

Adult mouse primary dorsal root ganglion (DRG) cultures were produced from either balb/c mice or balb/c SCID mice age >3 weeks. Two mice were used for one 12-well plate of culture.

2.3.3.1 Coating coverslips

25mm sterile coverslips were placed into a 12 well plate and covered with poly-DL-ornithine ((Sigma, UK), 100µg/mL in 0.15M boric acid) overnight. These were then washed 3 times with 1x PBS and covered with laminin ((Sigma, UK), 5µg/mL in 1x PBS) for 3-4 hours at 37°C.

2.3.3.2 Dissection and digestion of dorsal root ganglia

Mice were sacrificed using CO_2 and all DRGs extracted and placed into HBSS. The ganglia were then cleaned of blood and excess nerve with fine forceps and scalpel and placed into 1.6mL DMEM. 400µL of collagenase ((Roche, UK), 1.25% in 1x PBS) and 40µL trypsin (2.5%) were added to the ganglia and incubated at 37°C for 2 hours, agitating every 20 mins until fluffy in appearance. Ganglia were then washed twice in excess DMEM containing 10% horse serum (Gibco, Paisley, UK) to inactivate the collagenase.

2.3.3.3 Dispersion and plating of dorsal root ganglia

The ganglia were dispersed using Pasteur pipettes with diminishing diameter and then 12mL DRG media added (DMEM + 10% Horse serum) and the ganglia plated out onto the pre-prepared coated coverslips (after removing the laminin) at 1mL/well. After 24 hours the media was changed to DRG media supplemented with 5μM cytosine arabinoside to kill all dividing non-neuronal cells. After 2 days the cultures were ready for viral infection.

2.3.4 Transient transfection assays

2.3.4.1 DNA transfections

Transfections were performed with plasmid DNA prepared by the 'midi-prep' method (section 2.2.2.2). The transfection method was based on the calcium phosphate-mediated method (Stow and Wilkie 1976). Cells were grown in 35mm wells until they were 80% confluent. Two tubes were set up labelled A and B. Tube A contained 31μL 2M $CaCl_2$, 10μg plasmid DNA and 20μg herring sperm DNA (Sigma). Tube B contained 400μL HEBES transfection buffer. (140mM NaCl, 5mM KCl, 0.7mM Na_2HPO_4, 5.5mM D-glucose, 20mM Hepes, pH 7.05 with NaOH). The contents of tube A were carefully mixed by gentle pipetting and then added slowly to tube B drop-wise with gentle mixing. The mixture was then left for 40 mins to allow the DNA to precipitate. Media was removed from the cell monolayer and the precipitated DNA mixture was then added and incubated for 30 mins. After this time, 1mL FGM was added to the cells. After a further incubation of 4-5 hours, the cells were DMSO shocked by removing the transfection/media from the cells and washing twice with FGM. 1mL of ice-cold 25% (v/v) DMSO in HEBES transfection buffer was added and

left for 1.5 mins. The DMSO solution was removed and the cells washed twice with FGM. A final 2mL FGM was added to the cells before incubation at 37°C/5% CO_2.

2.3.4.2 Detection of β-Galactosidase by X-Gal staining

Media was removed from the cells and then washed twice with 1x PBS. Cells were then fixed with 0.1% (v/v) glutaraldehyde in 1x PBS for 10 mins at room temperature. The cells were washed with 1 x PBS before the addition of X-Gal solution (5mM $K_3Fe(CN)_6$, 5mM $K_4Fe(CN)_6.6H_20$, 1mM $MgCl_2$, 150µg X-Gal dissolved in DMSO in 1x PBS). Cells were then incubated at 37°C until blue colour seen. The X-Gal stain was removed and cells washed twice in 1x PBS before addition of 70% (v/v) glycerol and storage at 4°C.

2.3.4.3 β-Galactosidase activity assay

To quantify the level of β-galactosidase, the high-sensitivity β-Galactosidase assay kit (Stratagene, Netherlands) was used as per the manufacturer's instructions. Briefly, cells were washed with 1x PBS, lysis buffer added and the cells frozen at -20°C for 30 mins. The cell lysates were then harvested and centrifuged to remove the cell debris. The supernatant was then used in a reaction with the substrate chlorophenol red-β-D-galactopyranoside (CPRG) and then assayed in triplicate using a Dynex Optiplex plate reader at 595nm. Occasionally the lysate required a 1:10 dilution before assay.

2.3.5 Virus construction and propagation

All HSV-1 recombinant vectors were derived from the 17syn+ HSV-1 strain isolated in Glasgow (Brown *et al.* 1973) and the 1764 strain (Coffin *et al.* 1996a). The latter strain refers to 17syn+ deleted for both copies of ICP34.5 (MacLean *et al.* 1991b) and an inactivating mutation (12bp linker insertion) in the C-terminal transactivation domain of VP16 – *in*1814 mutation (Ace *et al.* 1989). The VP16 deficiency was complemented for

by the addition of hexamethylene bis-acetamide (HMBA) to the growth media, to a final concentration of 3mM. BHK or MAM49 cells were used for virus generation and propagation.

2.3.5.1 Homologous recombination transfections

Transfections were carried out as in section 2.3.4.1 except that 10-30µg of viral DNA (see section 2.2.7) was added to tube A and the transfection was left for 5-7 hours before DMSO shock. FGM containing 3mM Hexamethylene bisacetamide (HMBA) was added to the transfections at the final stage. The transfections were left for 3 –5 days until complete cytopathic effect (CPE) was observed and were then harvested and freeze-thawed. The cells were then titred (see section 2.3.5.2) and the efficiency of recombination determined by assaying for recombinants having lost or gained a reporter gene.

2.3.5.2 Viral plaque assay

Serial ten fold dilutions of a virus suspension (either harvested from homologous recombination or from a pure stock) were prepared in DMEM without FCS and plated onto 80% confluent 35mm wells of appropriate cells in a total volume of 500µl. The virus was allowed to adsorb for 60 mins at 37°C/5%CO_2 and then the monolayers were overlaid with 2mL of a 1:2 (v/v) mixture of 1.6% carboxymethylcellulose (CMC): FGM supplemented with 3mM HMBA. The cells were then incubated for a further 48 hours at 37°C/5% CO_2 and the number of plaques were counted in order to determine the titre of the virus in plaque forming units (pfu)/mL.

Cells expressing green fluorescent protein (GFP) required no pre-treatment and were visualised directly under an inverted fluorescent microscope at a wavelength of 520nm. Cells expressing β-galactosidase were stained with X-Gal (section 2.3.4.2) prior to plaque counting. If the virus contained no reporter gene, the cells were fixed with glutaraldehyde (section 2.3.4.2) and 1mL of a 0.05% (w/v) crystal violet in 20%

(v/v) ethanol solution added for 3 mins before washing, and then visualising of viral plaques.

2.3.5.3 Purification of viral recombinants by plaque selection

Titred transfection mixes were visualised by one of the methods described in section 2.3.5.2 and the identified plaques were picked from the cell monolayer using a P20 Gilson micropipette (set at 3µl). Selected plaques were transferred into 100µl SFM and frozen at -80°C. The plaque suspension was thawed and 10µl and 90µl used to infect wells of a 6 well plate containing cells at 80% confluency in 500µL SFM per well. Cells were left for 1 hour at 37°C/5% CO_2 and then overlaid with 2mL 1:2 (v/v) mixture of 1.6% carboxymethylcellulose (CMC): FGM supplemented with 3mM HMBA. Cells were then incubated for a further 48 hours at 37°C/5%CO_2. The plaque purification process was repeated until a pure population was obtained. When this was achieved a single plaque was used to infect one well of a 6 well plate and incubated until CPE was detected. The whole well was then harvested and its titre determined (section 2.3.5.2). This was then used as a master stock (MS) for large-scale propagation of the recombinant virus.

2.3.5.4 High titre viral stock production

Approximately 300µl of MS was used to infect 90% confluent cells in 80cm^2 flasks. Cells were infected with virus in SFM for 1 hour at 37°C/5%CO_2 after which cells were overlaid with FGM containing 3mM HMBA if necessary. Cells were harvested when complete CPE was observed. The virus was grown in 175cm^2 flask until enough was obtained to infect 10 x 245mm^2 plates of cells grown to 90% confluency. Each plate was infected with 5x10^6 pfu of virus stock suspension in a total volume of 50ml FGM supplemented with HMBA. Cells were harvested by freezing at -80°C, followed by thawing and pooling of the cell suspensions. Cells were centrifuged at 3500rpm for 45 mins to remove cell debris. To pellet the virus particles, the supernatant was spun at 12000rpm in a Sorvall RC 26 Plus Centrifuge using a SLA-1500 rotor for 2 hours at

4°C. The supernatant was removed and a small volume of SFM media added (50µl per 250ml cell suspension). The virus pellet was resuspended by shaking on an orbital shaker overnight at 4°C. 25µl and 50µl aliquots of the resuspended virus were stored in liquid nitrogen. The titre of the resuspended virus was then determined (section 2.3.5.2).

2.3.5.4.1 Filtration of viral stocks

If the virus in use was a replication incompetent virus it was filtered during high-titre production to remove complementing proteins present in the cell suspensions. This was carried out after removal of the cell debris and was performed using a 0.65 + 0.45 µM progressive filter, (Sartorius, Germany) attached to a Watson-Marlow pump. The filtered supernatant was then centrifuged at 12000rpm for 2 hours in a Sorvall RC 26 plus centrifuge using a SLA-1500 rotor and resuspended as described (section 2.3.5.4).

2.3.6 Growth curves

80% confluent 12-well plates of BHK cells were infected at an MOI of 0.1 in a final volume of 250µl of SFM. Virus was allowed to adsorb for 30-60 mins at 37°C/5%CO_2 and then the monolayers were overlaid with 500µl of FGM (supplemented with 3mM HMBA if necessary). Cells were harvested at 0, 4, 7, 22, 30 and 51 hours post-infection. Harvested samples were freeze-thawed to disrupt the cells and the yield of the virus was measured by plaque assay (section 2.3.5.2).

2.4 *IN VIVO* WORK

All experiments were carried out in accordance with the UK Animal Scientific Procedures Act 1986.

Balb/C mice were obtained from Harlan Laboratories (Oxon, UK). Balb/C SCID mice were obtained from breeding colonies within the Department of Biological Services, UCL. SCID mice are homozygous for the severe combined immune deficiency spontaneous mutation (*Prkdc*^{scid}, commonly referred as *SCID*) and are characterised by an absence of functional T and B cells.

2.4.1 Footpad delivery

Groups of three or ten mice were anaesthetised by fluothane inhalation and inoculated in the left rear footpad (Cook and Stevens 1973a; Stevens and Cook 1971a) with 20µl of a high titre stock (section 2.3.5.4) of recombinant vector, at the desired dose. At various times post-inoculation the mice were sacrificed and the ipsilateral lumbar DRG (L3-L6) were removed and reporter gene expression determined (sections 2.4.1.1 and 2.4.1.2).

2.4.1.1 Detection of β-Galactosidase activity in extracted DRG

DRG dissected from mice were placed in 1x PBS. The DRG were then fixed with 4% (w/v) paraformaldehyde (PFA) in 1x PBS for 1 hour on ice. The DRG were then washed 3 times with 1x PBS for 15 mins each wash. The DRG were then placed in 100µl of DRG X-Gal solution (5mM $K_3Fe(CN)_6$, 5mM $K_4Fe(CN)_6.6H_2O$, 1mM $MgCl_2$, 0.02% (w/v) sodium deoxycholate, 0.02% (v/v) NP-40 and 40mg/mL X-Gal in DMSO, in 1x PBS) and incubated at 37°C overnight. The X-Gal solution was removed and the DRG placed in 70% (v/v) glycerol and stored at 4°C prior to photography. DRG to be

photographed were mounted in 1x PBS on a glass slide and coverslipped. Photographs were taken at 10x magnification on a Zeiss Axiophot microscope.

2.4.1.2 Detection of GFP in extracted DRG

DRG dissected from mice were placed in 1x PBS. The DRG were then fixed with 4% (v/v) PFA in 1x PBS for 1 hour, on ice. The DRG were then washed 3 times with 1x PBS for 15 mins each wash. DRG to be photographed were mounted in 1x PBS on a glass slide and coverslipped. GFP expression was then visualised under fluorescent light (520 nm) at 10x magnification and photographed (Zeiss Axiophot microscope). When dual expression of GFP and β-Gal was to be assessed, GFP fluorescence was examined prior to X-Gal staining as otherwise β-Gal masked the GFP fluorescence.

2.4.2 Stereotaxic injections

For all CNS injections a Kopf stereotaxic frame (dual small animal stereotaxic, Kopf Instruments, USA) was used and coordinates established according to a stereotaxic mouse brain atlas (Franklin and Paxinos 1997). The head of the anaesthetised animal was positioned into the frame, and a sagittal incision was made to expose the skull. Tissue was removed and bregma and lambda points marked. Bregma was defined as the point of intersection of the sagittal suture with the curve of best fit along the coronal suture. When the two sides of the coronal suture met the sagittal suture at different points, bregma usually falls midway between the two junctions. The incisor bar was adjusted until the heights of lambda and bregma were equal (flat-skull position). The tip of the needle was positioned at Bregma, the appropriate anterior-posterior and medial-lateral coordinates calculated and the injection site marked. A hole was made through the skull with a needle without damaging dura. The injection needle was lowered until the tip just touched the tissue surface. The dorsal-ventral coordinates were calculated and the needle accordingly lowered. Injections were carried out using

an automatic micro injector (World Precision Instruments, USA) with a flow rate set at 0.5-1µL/min. Following injection, the needle was left in place for an additional 5 mins to allow the injectate to diffuse from the needle tip. The needle was then removed within 3mins. The wound was closed using metal clips and the animal allowed to recover. At various times post-inoculation the mice were sacrificed, perfused with ice-cold 4% (w/v) PFA and the brains removed. The brains were post-fixed for 1 hour in 4% PFA and then washed in 1x PBS.

2.4.2.1 Detection of β-Galactosidase activity in extracted brains

Whole brains were set in 3% agarose using peel-away agarose moulds (Agar Scientific, Cambs., UK), and then mounted with superglue onto a vibrating microtome (Campden Instruments, Loughborough, UK). Sections were taken from the injected side of the brain of 200µM thickness and then placed into X-gal tissue stain (see section 2.4.1.1). Sections were incubated overnight at 37°C to allow for colour development. Sections were then washed in 1x PBS and stored in 70% (v/v) glycerol until photographed.

2.5 STATISTICAL ANALYSIS

Statistical analysis was performed using the appropriate statistical test. Statistical comparisons were made by ANOVA when more than three groups were involved. Data were expressed as means ± standard error of the means (SEM). All statistics were performed using the Prism software package Version 4.03 (Graphpad Software Inc, CA, USA).

CHAPTER 3:

CONSTRUCTION AND TESTING OF HSV1 VECTORS CONTAINING LATP2 DELETION CONSTRUCTS (I)

3.1 INTRODUCTION

Long-term transgene expression from HSV1 vectors has been achieved by a number of different groups as discussed in chapter 1 (Andersen *et al.* 1992; Berthomme *et al.* 2000; Bloom *et al.* 1994; Bloom *et al.* 1995; Carpenter and Stevens 1996; Chattopadhyay *et al.* 2005; Dobson *et al.* 1989; Dobson *et al.* 1990; Goins *et al.* 1994; Goins *et al.* 1999; Ho and Mocarski 1989; Lachmann and Efstathiou 1997; Lokensgard *et al.* 1994; Lokensgard *et al.* 1997; Marshall *et al.* 2000; Perez *et al.* 2004; Puskovic *et al.* 2004). It has been shown that the region downstream of the LAP2 promoter, the long-term expression element (LTE), is required to maintain expression from the LAP1 promoter during latency (Berthomme *et al.* 2000; Lachmann and Efstathiou 1997; Lokensgard *et al.* 1997).

Latency associated transcript promoter 2 (LATP2) is the 1.35kb stretch downstream of the LAP1 promoter, encompassing the LAP2 promoter and the LTE (nt 118866 to 120219). It contains promoter, enhancer and long-term expression elements, the latter two of which can work in a bi-directional manner. It has been used successfully to confer long-term expression on the LAP1 promoter from the gC locus (Berthomme *et al.* 2000; Lokensgard *et al.* 1997) and the U_L43 locus (Palmer *et al.* 2000). The LATP2 region and its relationship to the rest of the genome are shown in figure 3-1.

The bi-directional function of LATP2 has been exploited in the previously described vector from our laboratory, 1764/U_L43/pR20.9. See figure 3-2 (Palmer *et al.* 2000). This vector contains a cassette with the LATP2 region between LAP1 and the MMLV LTR driving genes for *lacZ* and GFP, respectively. When placed in the non-LAT U_L43 locus, within a replication competent viral backbone (1764), this arrangement of LATP2 and promoters has been shown to give transgene expression for at least six months post-infection in the mouse PNS (unpublished results). The fact that this vector can drive expression of two exogenous genes means that it has the potential to be a

useful tool for a number of research and gene therapy applications in the nervous system, where delivery of more than one therapeutic gene is required.

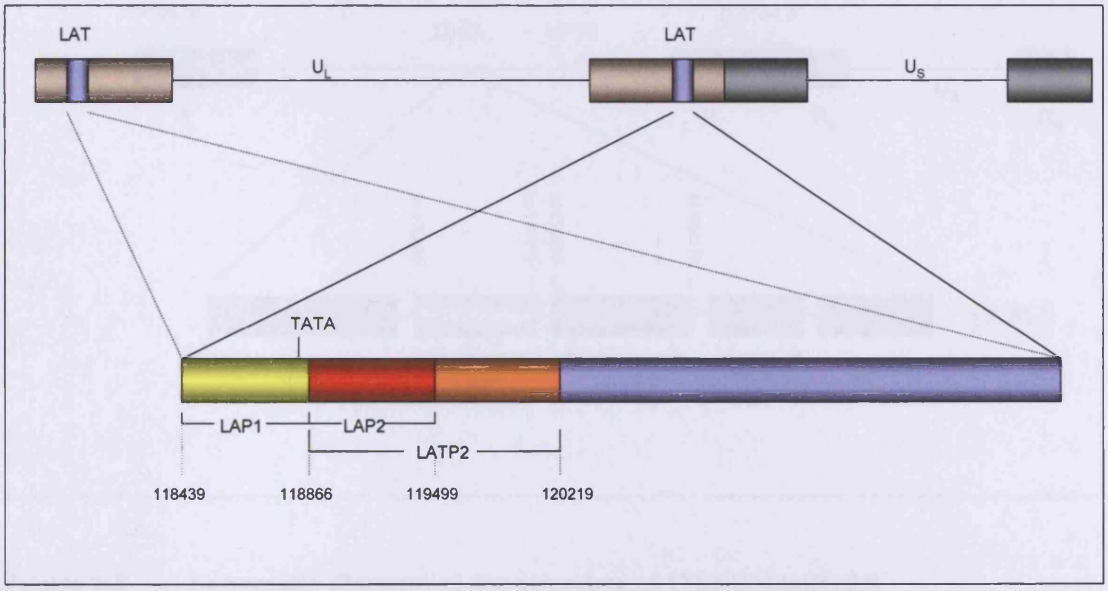


Figure 3-1 Diagram of the LATP2 region and the surrounding genome

Numbering refers to nucleotide positions based on the published 17*syn*+ genomic sequence.(McGeoch *et al.* 1986; McGeoch *et al.* 1988; McGeoch *et al.* 1991; Perry and McGeoch 1988). U_L = unique long region. U_S = unique short region.

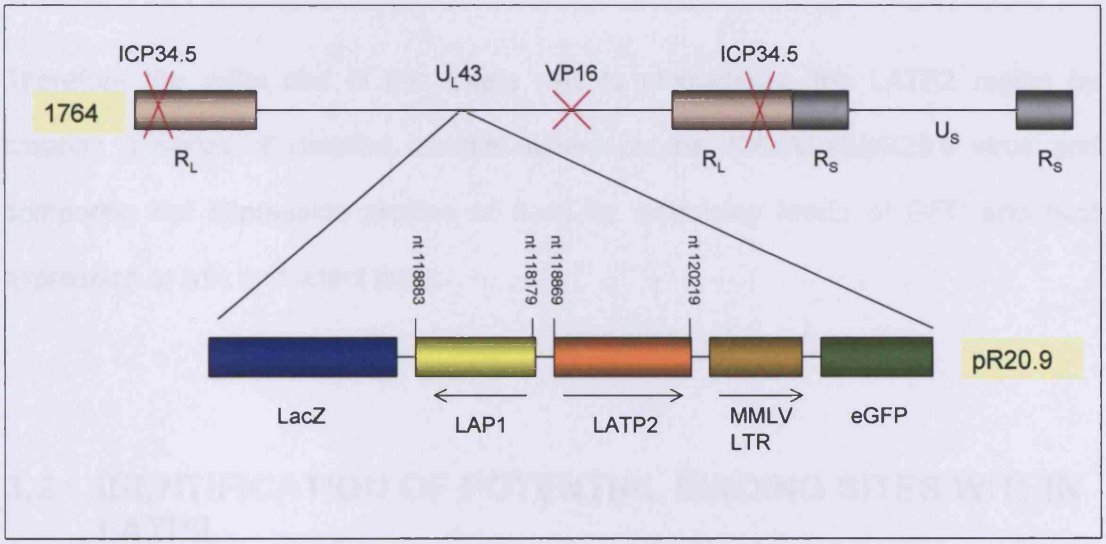


Figure 3-2 Schematic diagram of the structure of 1764/U_L43/pR20.9

The deletions in the backbone virus are shown along with the site of insertion of the cassette into the virus (U_L43 gene). Arrows show the orientation of the promoter elements in the cassette. R_L & R_S = Long and short repeat regions. U_L & U_S = Long and short unique regions.

An important aspect of the development of HSV-based vectors for long-term neuronal delivery is the identification and characterisation of promoter and regulatory elements that can drive the stable long-term expression of foreign genes during neuronal latency. Although the LATP2 region has been shown to be required for long-term expression, it has not been fully characterised, although since this project began, other groups have attempted to do this (Berthomme *et al.* 2001). Identifying which regions of LATP2 contain regulatory elements could lead to optimal long-term expression from this region and thus improved gene therapy vectors.

Therefore the initial aim of this thesis was to characterise the LATP2 region by creating a series of deletion mutants based on the 1764/U_L43/pR20.9 virus and comparing the expression profiles of each by examining levels of GFP and *lacZ* expression at lytic and latent times.

3.2 IDENTIFICATION OF POTENTIAL BINDING SITES WITHIN LATP2

It is already known that the LAP2 promoter contains potential *cis*-acting elements that may be responsible for modulating the promoter activity. These include an Sp1 site and putative AP2 and E2F sites (Goins *et al.* 1994). LAP2 also contains CT rich and polyT elements that bind transactivating factors known to regulate cellular housekeeping gene promoters.

The program TFSEARCH (Heinemeyer *et al.* 1998), was used to search the whole LATP2 sequence (nt 118869-120219 from HSV1 genbank accession number X14112) for other possible transcription factor (TF) binding sites. The results are shown in figure 3-3.

A number of consensus elements for transcription factors were identified, most abundantly for AML-1a and GATA. AML-1a is known to be a transcriptional activator and binds to enhancer motifs (Meyers *et al.* 1993) whilst GATA TFs are transcriptional activators (Merika and Orkin 1993). Other putative binding sites for TFs include p300 which possesses histone acetyltransferase activity, which usually activates transcription (Ogryzko *et al.* 1996) but can also act as a co-repressor (Guidez *et al.* 2005); MZF1 which has been shown to be a transcriptional repressor (Gaboli *et al.* 2001; Morris *et al.* 1994) and ZID which is also thought to be involved in transcriptional repression by interacting with histone deacetylases and Sp1.

This suggests that the region contains both positive and negative regulatory elements and as such by creating deletion mutants of the region it will hopefully be possible to identify where the strongest repressing or activating elements are located.

Start of 5' half LATP2/LAP2

118869 c cggggcggcc aaggggcgtc ggcgacatcc tccccctaag cgccggccgg

118921 ccgctggtct gttttttcgt tttccccgtt tcgggggtgg tgggggttgc ggtttctgtt

118981 tctttaaccc gtctggggtg tttttcgttc cgtcgccgga atgtttcgtt cgtctgtccc

119041 ctcacggggc gaaggccgcg tacggcccgg gacgaggggc ccccgaccgc ggcggtccgg

Start of Mid LATP2

119101 gccccgtccg gacccgctcg ccggcacgcg acgcgaaaaa ggccccccgg aggcttttcc

119161 gggttcccgg cccggggcct gagatgaaca ctcggggtta ccgccaacgg ccggcccccg

119221 tggcggcccg gcccggggcc ccggcggacc caaggggccc cggcccgggg ccccacaacg

119281 gcccggcgca tgcgctgtgg tttttttttc ctcggtgttc tgccgggctc catcgccttt

119341 cctgttctcg cttctccccc ccccttctt caccccagt accctcctcc ctcccttcct

119401 ccccccgttat cccactcgtc gagggcgccc cggtgtcgtt caacaaagac gccgcgtttc

Start of 3' half /end of 5' half LATP2/LAP2

119461 caggtaggtt agacacctgc ttctccccaa tagaggggggg ggacccaaac gacaggggc

119521 gccccagagg ctaaggtcgg ccacgccact cgcgggtggg ctcgtgttac agcacaccag

119581 cccgttcttt tccccccctc ccacccttag tcagactctg ttacttaccc gtccgaccac

End of Mid LATP2

119641 caactgcccc cttatctaag ggccggctgg aagaccgcca gggggtcggc cggtgtcgct

119701 gtaacccccc acgccaatga cccacgtact ccaagaaggc atgtgtccca ccccgcctgt

119761 gtttttgtgc ctggctctct atgcttgggt cttactgcct gggggggggg agtgcgggggg

119821 agggggggtg tggaaggaaa tgcacggcgc gtgtgtaccc cccctaaagt tgttcctaaa

119881 gcgaggatac ggaggagtgg cgggtgccgg gggaccgggg tgatctctgg cacgcggggg

119941 tgggaagggt cgggggaggg ggggatggag taccggccca cctggccgcg cgggtgcgcg

120001 tgcctttgca caccaacccc acgtcccccg gcggtctcta agaagcaccg ccccccctcc

120061 ttcataccac cgagcatgcc tgggtgtggg ttggtaacca acacgcccat cccctcgtct

120121 cctgtgattc tctggctgca ccgcattctt gttttctaac tatgttcctg tttctgtctc

End of 3' half LATP2

120181 ccccccccccc acccctccgc cccacccccc aacacccac

Figure 3-3 **Sequence of LATP2 showing putative TF binding sites.**
TF binding sites with a score of >90 are shown from a search using the TFSEARCH program. The areas of LATP2 used for cloning are also marked.

Key:

AML-1a	MZF1	AhR/Ar	v-Myb	c-Myb
GATA-1	ZID	Sp1	p300	

3.3 LATP2 DELETION PLASMIDS

A series of plasmids was constructed based on the pR20.9 plasmid with various deletions of the LATP2 region (figure 3-4). The first contained the 3' half of LATP2, which removes the LAP2 promoter. The second contained the mid section of LATP2 with regions from both the 3' and 5' ends deleted. The third contained the 5' half of LATP2, equating to the LAP2 promoter only. A fourth plasmid was created by removing the LATP2 region from pR20.9 completely. Details of construction are provided below.

The reporter genes used in this study were green fluorescent protein (GFP) and *lac*Z. The *lac*Z gene encodes the bacterial *E. coli* enzyme *lac*Z (β-Gal), which forms a blue intracellular precipitate upon incubation with "X-Gal" substrate. This reporter gene is commonly used to detect gene expression in neural tissue (Sanchez-Ramos *et al.* 2000), and elsewhere. GFP use is also widespread. GFP is very stable, relatively non-toxic and allows direct detection of gene expression. Each of these properties makes GFP a very useful marker gene (Tsien 1998). Zhang and co-workers developed an enhanced GFP protein (eGFP) (Zhang *et al.* 1996). This version gives enhanced fluorescence compared to wild type GFP and is widely used to detect gene transfer in mammalian systems. The eGFP version from pEGFPNI (Clontech) was used throughout this thesis.

3.3.1 Method of Construction

pR20.9 was digested with *Kpn*1/*Nsi*1 to remove the LAP1/LATP2/MMLV/GFP fragment. LAP1 was digested from pP1/*lacZ*Srf with *Kpn*1/*Nsi*1 and re-ligated into the digested pR20.9 plasmid.

An oligonucleotide was then ligated after LAP1, to allow reinsertion of MMLV & GFP (from pJ4GFP (Morgenstern and Land 1990) by digestion with *Nsi*1/T4/*Nhe*1):

Nsil compatible ends

XhoI *BspEI* *SpeI* *NheI* *HindIII*

Strand 1 - **5' CTCGAGTCCGGACTAGTGCTAGCTGACAAGCTTTGCA 3'**

Strand 2 – **5' AAGCTTGTCAGCTAGCACTAGTCCGGACTCGAGTGCA 3'**

The LATP2 deletion fragments were all digested from pP2BSK (M. Robinson) as follows: -

(1) *PpuM*1 – *Spe*1 (HSV nt 119502 - 120219) (3'LATP2)

(2) *BspE*1 – *Bbs*1 (HSV nt 119107 – 119677) (Mid LATP2)

(3) *Xho*1 – *PpuM*1 (HSV nt 118866 – 119502) (5' LATP2)

(4) No LATP2

These were then ligated into the cassette at the oligonucleotide by digestion with appropriate enzymes to give the series of LATP2 deletion plasmids (Figure 3-4).

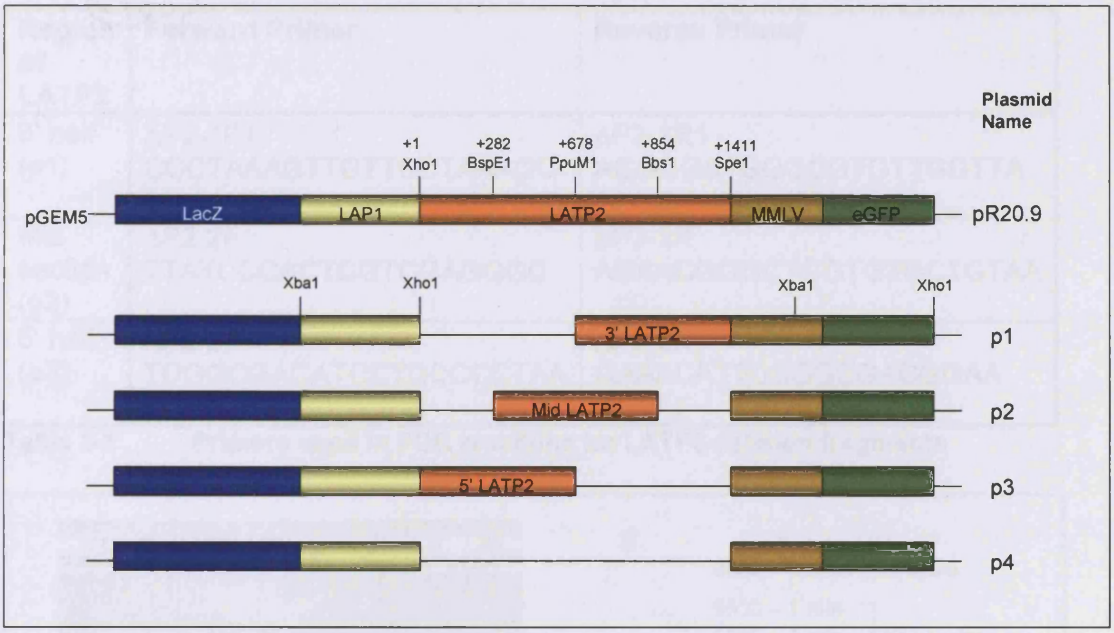


Figure 3-4 Schematic of LATP2 deletion cassettes

Restriction sites used to clone LATP2 fragments from pP2BSK are shown along with numbering relative to the start of LATP2 in the same plasmid. Other restriction sites shown were used in restriction digest analysis (NB Diagram not to scale).

Plasmid structures were confirmed by restriction digest analysis using *Xba1* or *Xho1*. PCR was also carried out to confirm that the correct fragments of LATP2 were present. Primers exclusive to each section of LATP2 were used in PCR reactions on all five plasmids as shown in Table 3-1 and Figure 3-5.

Region of LATP2	Forward Primer	Reverse Primer
3' half (p1)	ΔP2-1F1 CCCTAAAGTTGTTCCTAAAGC	ΔP2-1R1 AGGGGATGGGCGTGTTGGTTA
Mid section (p2)	ΔP2-2F TTATCCCACTCGTCGAGGGC	ΔP2-2R AGAACGGGCTGGTGTGCTGTAA
5' half (p3)	ΔP2-3F TCGGCGACATCCTCCCCCTAA	ΔP2-3R GAAACATTCCGGCGACGGAA

Table 3-1 Primers used in PCR reactions for LATP2 deletion fragments

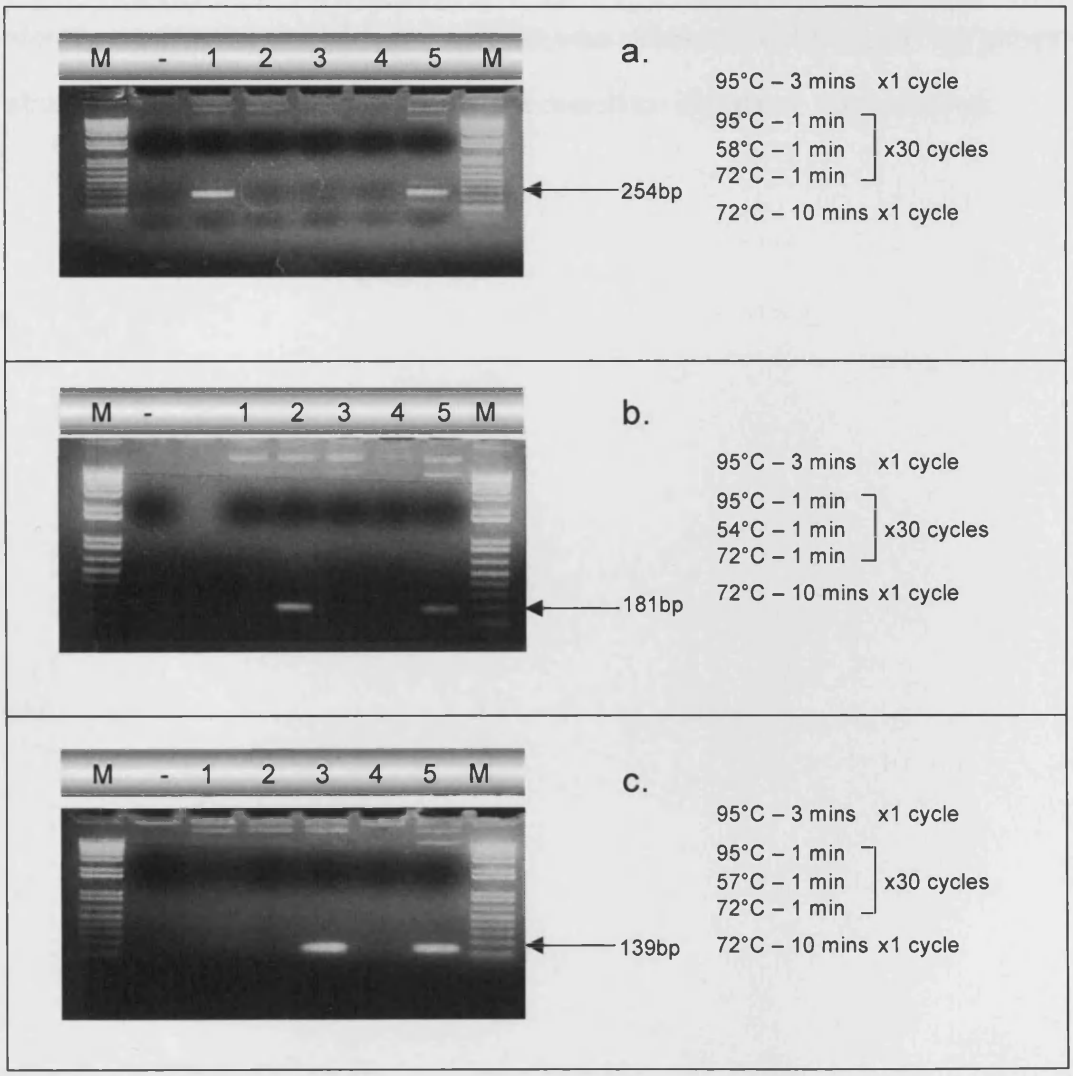
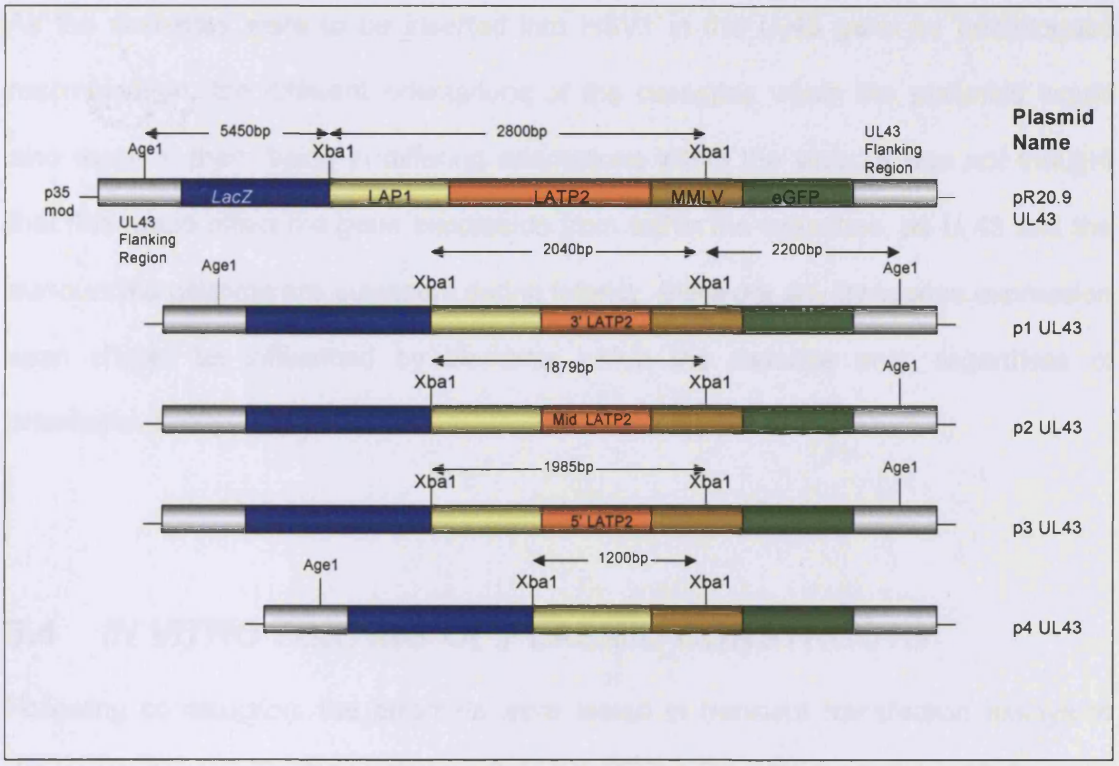


Figure 3-5 Gel photos showing PCRs on all five plasmid constructs with primers for each section of LATP2 and the respective PCR program used.
a - Primers for 3' half LATP2. b - Primers for mid LATP2. c - Primers for 5' half LATP2. (M = Marker, 1 = p1, 2 = p2, 3 = p3, 4 = p4, 5 = pR20.9)

The cassettes were then excised from the pGEM5 (Promega) backbone with *Srf*1 and ligated into the plasmid p35mod at the unique site *Nsi*1. p35mod contains a 5.1Kb *BamH1/EcoR*1 fragment of HSV1 (nt 91619-96751), encompassing the U_L43 gene and regions flanking U_L43, inserted into pGEM2 (Promega). Insertion into the *Nsi1* site in the U_L43 gene (nt 94911) causes inactivation of the gene. Use of this plasmid will allow recombination of the cassettes into the U_L43 gene of HSV1.

Because the insertion into p35mod can occur in either orientation due to the double-blunt digestion and ligation necessary, restriction digests of the resulting plasmids were carried out firstly to check that the insertion had taken place and secondly to identify in which orientation each cassette was relative to U_L43. Figure 3-6 shows the structure of the cassettes in U_L43 and the restriction digests by *Xba*1 and *Age*1.



a.

b.

Figure 3-6 Structure of the LATP2 deletion cassettes in U_L43 flanking regions

a. Diagram showing the structure of the different plasmids and the restriction sites used for analysis. The position of the *Age*1 site differs between plasmids as the cassettes were inserted into the U_L43 region by double-blunt ligation.

b. Gel photo of restriction digests using *Xba*1 and *Age*1 to show orientation of the cassettes. (M = Marker. 1 = p1/U_L43, 2 = p2/U_L43, 3 = p3/U_L43, 4 = p4/U_L43, 5 = pR20.9/U_L43) It can be seen that cassettes 1, 2 and 3 are in the opposite orientation to cassettes 4 and 5.

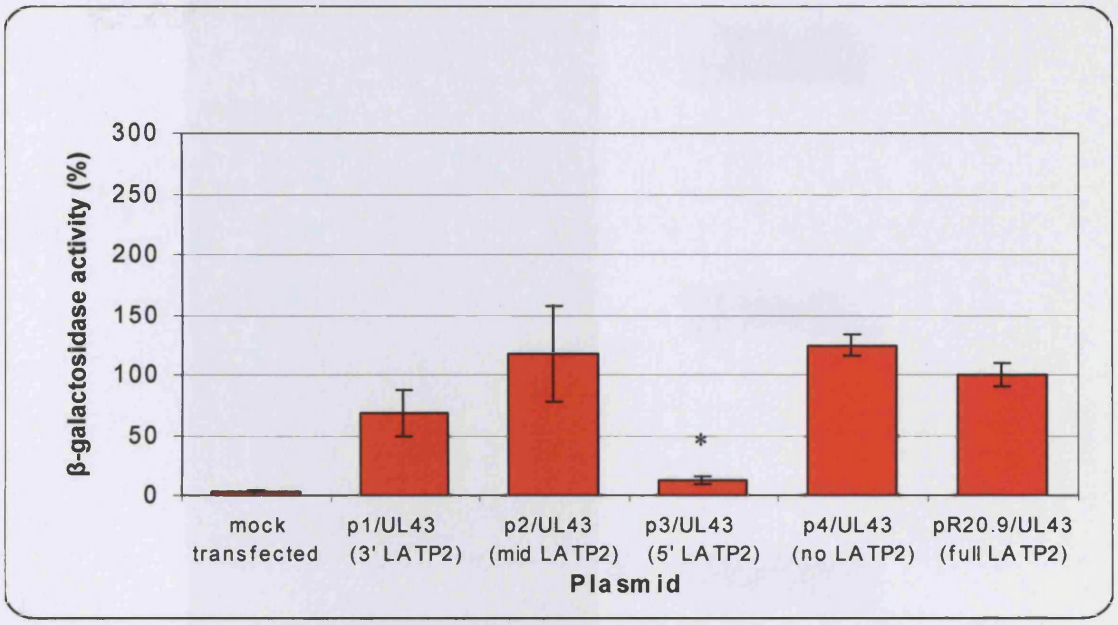
As the cassettes were to be inserted into HSV1 in the U_L43 gene by homologous recombination, the different orientations of the cassettes within the plasmids would also result in them being in differing orientations within the virus. It was not thought that this would affect the gene expression from within the cassettes, as U_L43 and the surrounding genome are quiescent during latency, therefore any transgene expression seen should be influenced by elements within the cassette only, regardless of orientation in the virus.

3.4 *IN VITRO* TESTING OF PLASMID CONSTRUCTS

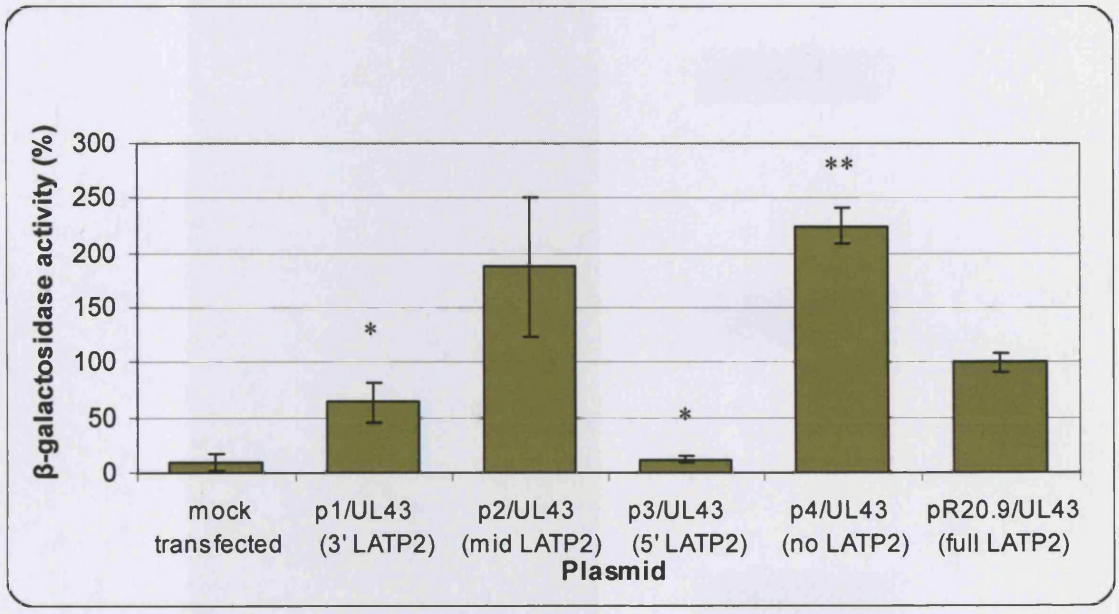
Following construction, the plasmids were tested in transient transfection assays to determine the levels of expression of the marker genes. These were carried out in BHK cells (fibroblast) and ND7 cells (neuronal).

5μg of each plasmid was transfected into each cell line (n=4). In 3 sets of the transfections the cells were harvested after 48 hours and the level of *lacZ* expression determined using the high-sensitivity β-Galactosidase assay kit (Stratagene), (i.e. the results of 9 individual transfections). The level of expression for each plasmid was expressed as a percentage of the total activity of pR20.9/U_L43. The results are shown in figure 3-7.

The fourth set of transfections was examined after 48 hours for GFP expression under a fluorescence microscope in order to visualise this expression. The levels of GFP were not quantified due to inaccuracies in counting GFP positive neurons under a microscope. Examples of GFP expression in the transfected cells are shown in figure 3-8a-b.



a. BHK



b. ND7

Figure 3-7 ***LacZ* activity of LATP2 deletion plasmids *In Vitro*.**

5μg plasmid was transfected into BHK cells (a) and ND7 cells (b). Data is represented as the % *lacZ* activity compared to that given by pR20.9/U_L43. The graphs depict average values of 3 transfection wells and the error bars are shown as SEM. (* - $p<0.05$, ** - $p<0.01$)

Figure 3-8a **GFP expression from plasmids in BHK cells.**

Cells were transfected with 5μg each plasmid and after 48 hours visualised for GFP expression. The photographs are representative regions of transfections used for the graphs in figure 3-7.

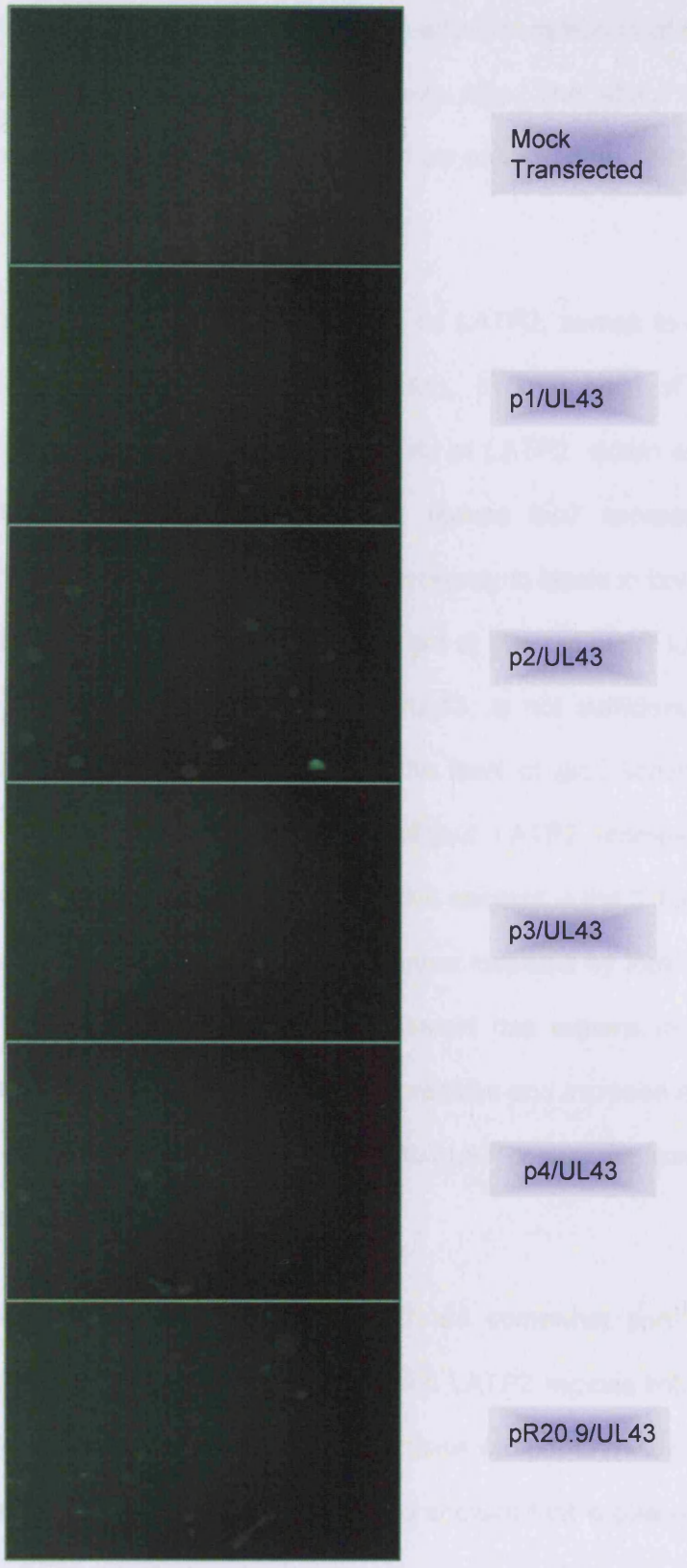


Figure 3-8b GFP expression from plasmids in ND7 cells.

Cells were transfected with 5μg each plasmid and after 48 hours visualised for GFP expression. The photographs are representative regions of transfections used for the graphs in figure 3-7.

The results of the *lacZ* quantification show the effect of deletions of the LATP2 region on *lacZ* expression from plasmid constructs *in vitro.* The effect of LATP2 in this arrangement would be expected to be that of an enhancer on LAP1 promoting *lacZ* expression.

Interestingly, p4/U_L43, the plasmid containing no LATP2, seems to give a similar (in BHK cells) or significantly higher (in ND7 cells, $p<0.01$) level of *lacZ* expression compared to pR20.9/U_L43, containing the whole of LATP2. When only the 5' half of LATP2 (p3/U_L43) is present, it appears to reduce *lacZ* expression from LAP1 significantly ($p<0.05$). It is almost reduced to background levels in both cell types. This could suggest that there is a repressive element in this region of LATP2. Removing this 5' region, as is the case in plasmid p1/U_L43, is not sufficient to alleviate this repression fully, particularly in ND7s where the level of *lacZ* activity is significantly ($p<0.05$) lower compared to the plasmid without LATP2 (compare p1/U_L43 and p4/U_L43). This suggests that there is a repressive element in the 3' half of LATP2 also. These repressive elements can perhaps be further localised by looking at the level of *lacZ* expression in plasmid p2/U_L43. This plasmid has regions from both ends of LATP2 deleted which seems to remove the repression and increase enhancer activity, allowing *lacZ* expression above that from the full LATP2 and approximately to that of the plasmid containing no LATP2 (p4/U_L43).

The results from the plasmid with no LATP2 are somewhat surprising, as similar studies have shown that plasmids containing full LATP2 regions linked to LAP1 give much higher (4 – 5x) expression of *lacZ* than those without LATP2 (Berthomme *et al.* 2000; Berthomme *et al.* 2001). This group also showed that a plasmid containing the 5' half of the LTE gave similar *lacZ* expression to that of a plasmid containing the whole LATP2 region. However, in both these cases, the LATP2 region was placed downstream of LAP1, between LAP1 and the *lacZ* gene, so as to preserve the arrangement of the LAT region to that of the native virus. This LAT promoter

arrangement and the fact that the plasmids used here also contain the MMLV LTR probably accounts for the difference in results seen.

3.5 VECTOR CONSTRUCTION

Viral vectors were produced using the 1764 virus backbone, as this is the virus that was used previously to insert the pR20.9/U_L43 cassette into for *in vivo* testing and allows long-term expression in the PNS. The 1764 viral mutant is deleted for the neurovirulence factor ICP34.5 and has an inactivated VP16 gene (see section 1.1.4 and figure 3-2), therefore having reduced toxicity compared to wild-type HSV1. This vector has previously been shown to infect the PNS efficiently in animal models (Coffin *et al.* 1996b; Palmer *et al.* 2000). The recombinant vector does not need to be grown on a complementing cell line, as ICP34.5 is non-essential for replication in cell culture. The VP16 mutation can be complemented for by addition of HMBA to the media (Ace *et al.* 1988).

The U_L43 gene has been shown to be a suitable insertion site for the expression cassettes from 1764. U_L43 has no known function, but contains multiple hydrophobic stretches and is predicted to be a membrane channel protein (Carter *et al.* 1996). U_L43 is not required for replication of the virus, nor does it affect the growth characteristics of the virus (MacLean *et al.* 1991a). The use of an insertion site outside of the LAT region assures that the virus establishes a normal latent infection by producing LATs. Deletion of certain regions of the LAT region may otherwise alter the ability to establish latency otherwise by altering LAT production as discussed in chapter 1, section 1.1.2.4.2 (Thompson and Sawtell 1997).

The four LATP2 deletion plasmids were linearised with *Xmn*1 and co-transfected with 1764 viral DNA (see figure 3-9). Recombinant vectors containing the cassettes were identified by gain of eGFP and *lacZ* marker genes and plaque purified. Insertion of the cassettes was confirmed by Southern blot analysis (see figure 3-10).

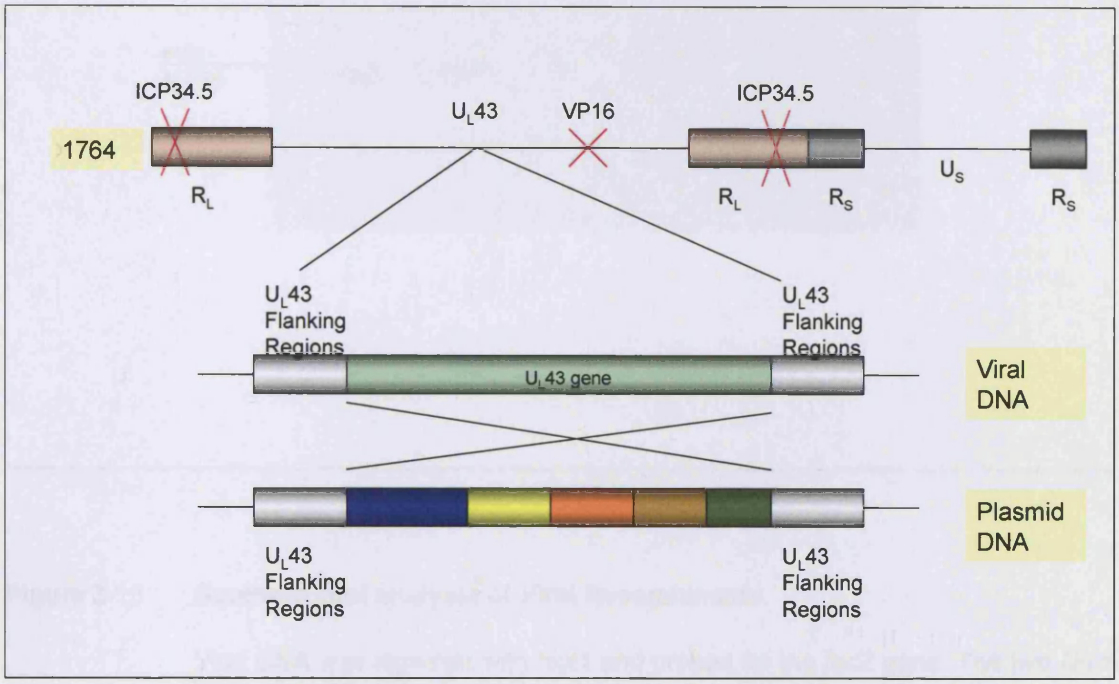


Figure 3-9 Homologous recombination to produce recombinant HSV vectors

Plasmid DNA was co-transfected with 1764 viral DNA into BHK cells. During lytic replication, homologous recombination occurs between the U_L43 flanking regions present in both plasmid and viral DNA.

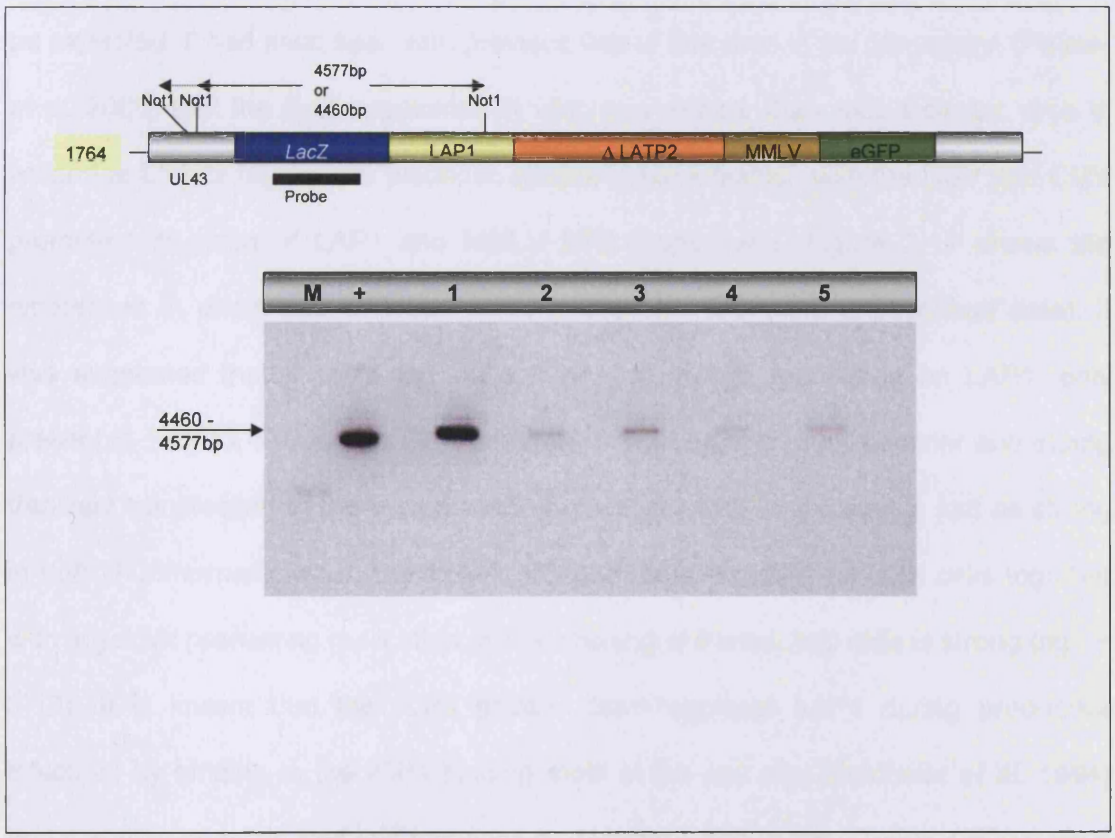


Figure 3-10 Southern Blot analysis of Viral Recombinants

Viral DNA was digested with *Not*1 and probed for the *lacZ* gene. The two *Not*1 sites in U_L43 are of an almost equal distance either side of the inserted cassettes such that either orientation of cassette in the virus gives a similar size band.
(M = 1Kb+ Marker, + = Positive plasmid control, 1 = 1764/U_L43/1, 2 = 1764/U_L43/2, 3 = 1764/U_L43/3, 4 = 1764/U_L43/4, 5 = 1764/U_L43/20.9)

3.6 *IN VITRO* TESTING OF RECOMBINANT VECTORS

3.6.1 Viral plaque characteristics

During purification of the viral vectors it was noted that the plaque phenotype of all of the LATP2 deletion mutants was similar to that of the 1764/U_L43/20.9 virus as would be expected. It had been seen with previous use of this virus in our laboratory, (Palmer *et al.* 2000) that the *lacZ* expression *in vitro* was weaker than with a similar virus in which the LATP2 region was placed in a back-to-back fashion with the RSV and CMV promoters in place of LAP1 and MMLV LTR respectively. Figure 3-11 shows the differences in phenotype between these viruses (R. Branston, unpublished data). It was suggested that it could be a result of viral protein repression on LAP1, only present in 1764/U_L43/20.9, as GFP expression from both viruses is similar and during transient transfection of the corresponding plasmids, *lacZ* expression is just as strong in both. Furthermore, when the 1764/U_L43/20.9 virus is plated on BHK cells together with acyclovir preventing replication, X-Gal staining of transduced cells is strong (figure 3-12). It is known that the ICP4 protein down-regulates LAP1 during productive infection, by binding to the ICP4 binding motif in the cap site (Batchelor *et al.* 1994) and it is thus possible that this functions to dampen LAP1 in the 1764/U_L43/20.9 virus *in vitro*. Nevertheless, *lacZ* expression from 1764/U_L43/20.9 in latently infected neurons *in vivo* is known to be strong (Palmer *et al.* 2000), perhaps due to an absence of ICP4 at this time.

Figure 3-12 shows the LATP2 deletion virus plaques expressing *lacZ* and GFP. It can be seen that all the LATP2 deletion viruses have the same phenotype as 1764/U_L43/20.9, with *lacZ* expression being repressed in the same manner. GFP expression appears to be similar between all viruses. The relief from repression given by acyclovir on lacZ expression is also illustrated.

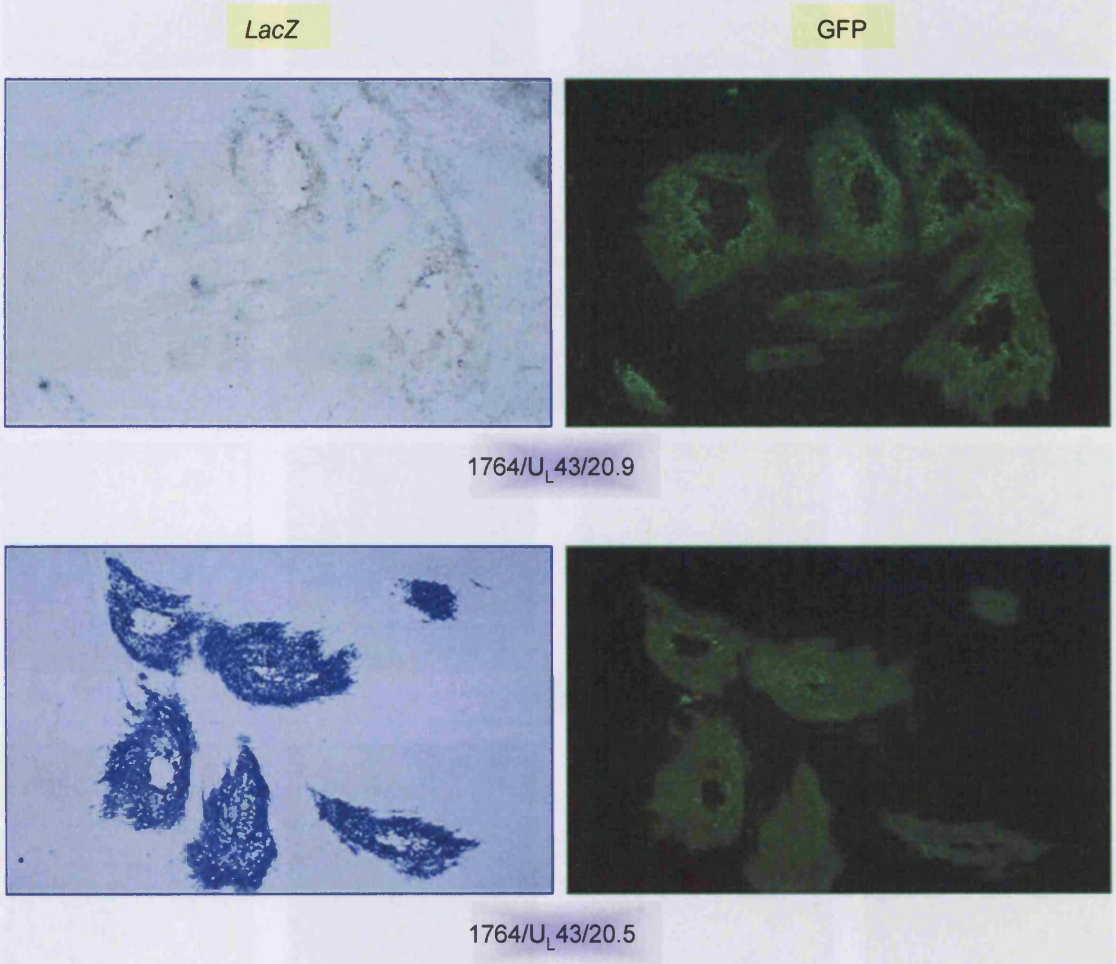


Figure 3-11 **Plaque phenotype of two replication competent vectors.**

Each vector contains the central LATP2 region in a back-to-back orientation with either LAP1 (20.9) or RSV (20.5) driving *lacZ* expression and MMLV LTR (20.9) or CMV (20.5) driving eGFP expression.
(Photographs courtesy of Dr. R. Branston.)

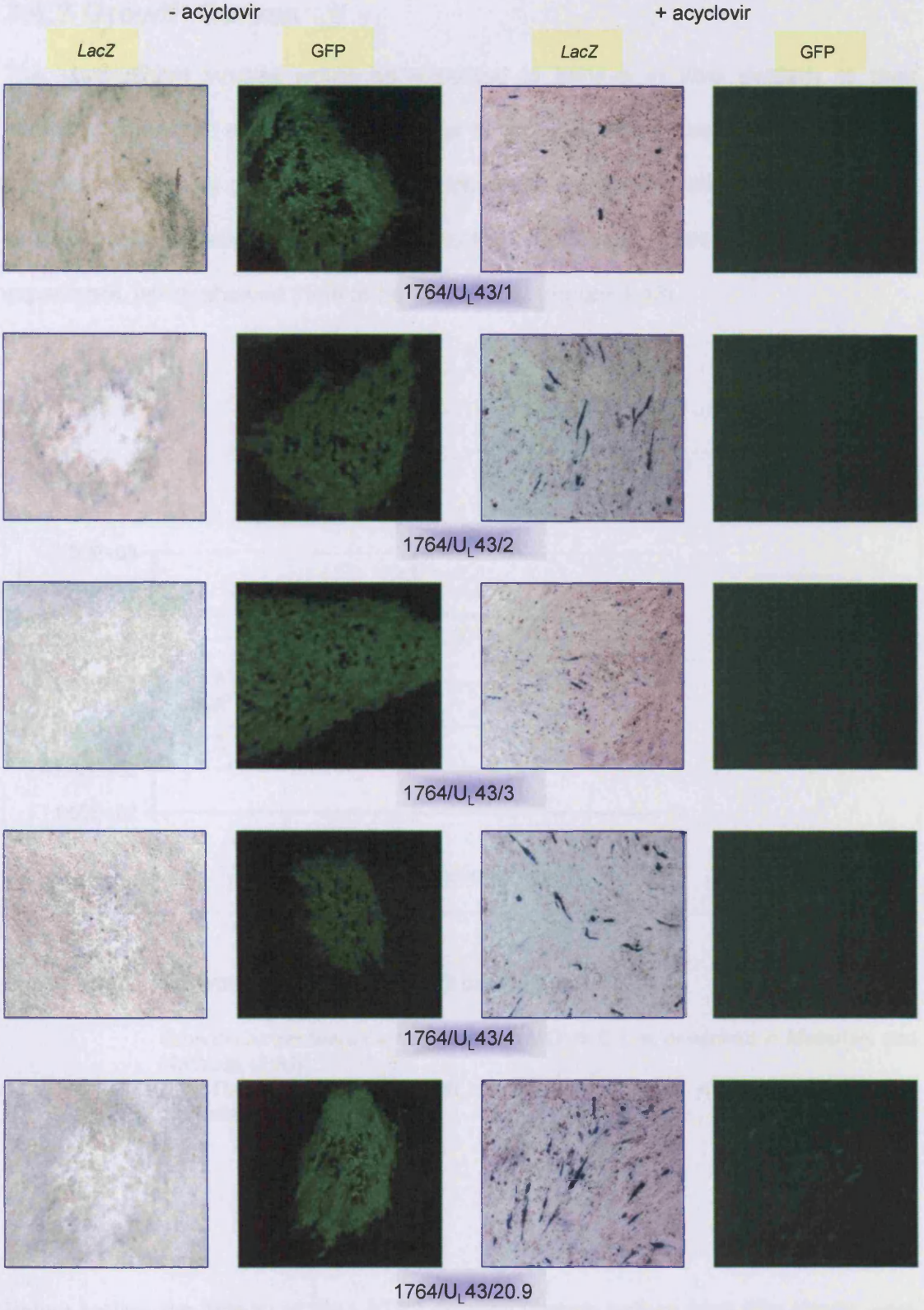


Figure 3-12 LATP2 deletion virus plaques showing *lacZ* and GFP expression

BHK cells were infected with virus + or - acyclovir and left for 48 hours. The GFP expression was then photographed before staining with X-Gal to visualise and photograph *lacZ* expression. Note that addition of acyclovir inhibits replication and thus plaque formation.

3.6.2 Growth Curves

The recombinant viruses would be expected to behave *in vitro* similarly to their parental counterpart since they contain just an insertion of a cassette into U_L43, whilst the rest of the viral genome remains unchanged. To confirm this, the recombinant viruses' growth kinetics were compared to the parent virus, 1764, in a growth curve experiment, which showed them to be very similar (Figure 3-13).

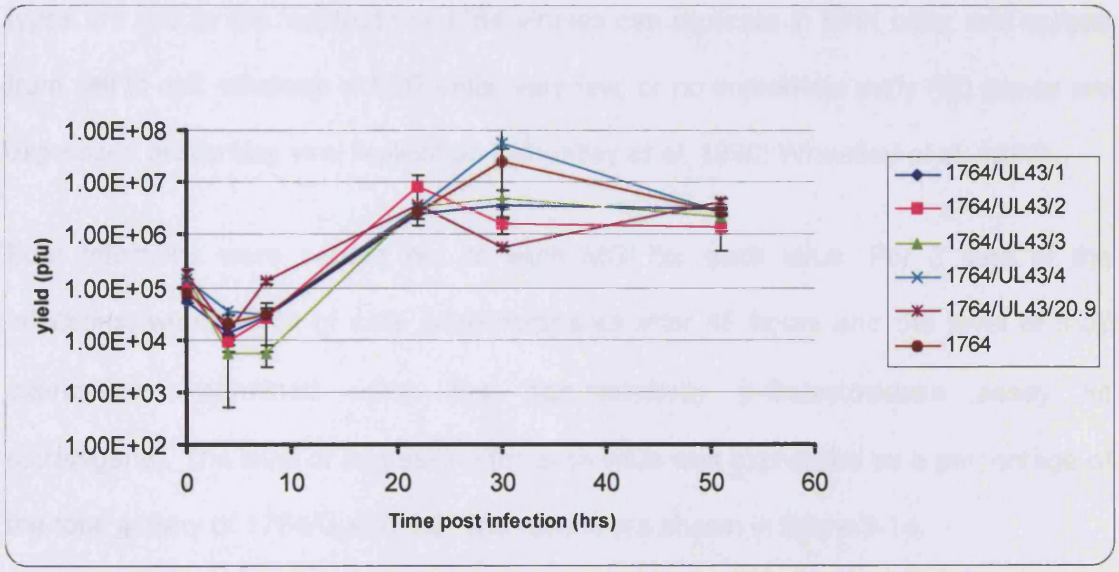


Figure 3-13 Growth Curves of the LATP2 deletion viruses

Growth Curves were carried out at an MOI of 0.1 as described in Materials and Methods (2.3.6).
N.B. The drop in titre between infection and 10 hours reflects virus entry into the cells.

Before testing the activity of the LATP2 deletion vectors further, high titre stocks were prepared (see 2.3.5.4), along with a stock of the 1764/U_L43/20.9 virus for use as a control.

3.6.3 Infection of cell lines

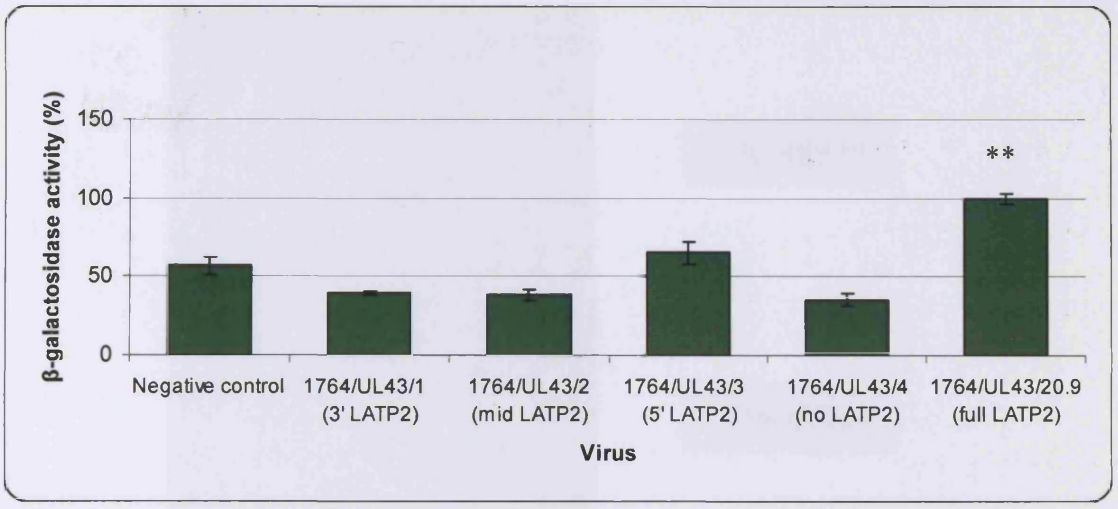
In order to determine the level of reporter gene expression occurring during lytic infection of non-neuronal BHK cells and neuronal ND7 cells, these two cell lines were infected with the viruses. 3 different MOIs were used for infection – 0.1, 1 and 5. In BHK cells, infections at an MOI of 1 or 5 caused considerable cell lysis, resulting in very low levels of measurable *lacZ* expression (data not shown). In ND7 cells, infections at an MOI of 0.1 resulted in inadequate infection of cells, also leading to non-quantifiable levels of *lacZ* and therefore no data. These differences between cell-types are due to the fact that the 1764 viruses can replicate in BHK cells, and spread from cell to cell, whereas in ND7 cells, very few, or no immediate early (IE) genes are expressed preventing viral replication (Wheatley *et al.* 1990; Wheatley *et al.* 1991).

Four infections were carried out at each MOI for each virus. For 3 sets of the infections, whole wells of cells were harvested after 48 hours and the level of *lacZ* expression determined using the high-sensitivity β-Galactosidase assay kit (Stratagene). The level of expression for each virus was expressed as a percentage of the total activity of 1764/U_L43/20.9. The results are shown in figure 3-14.

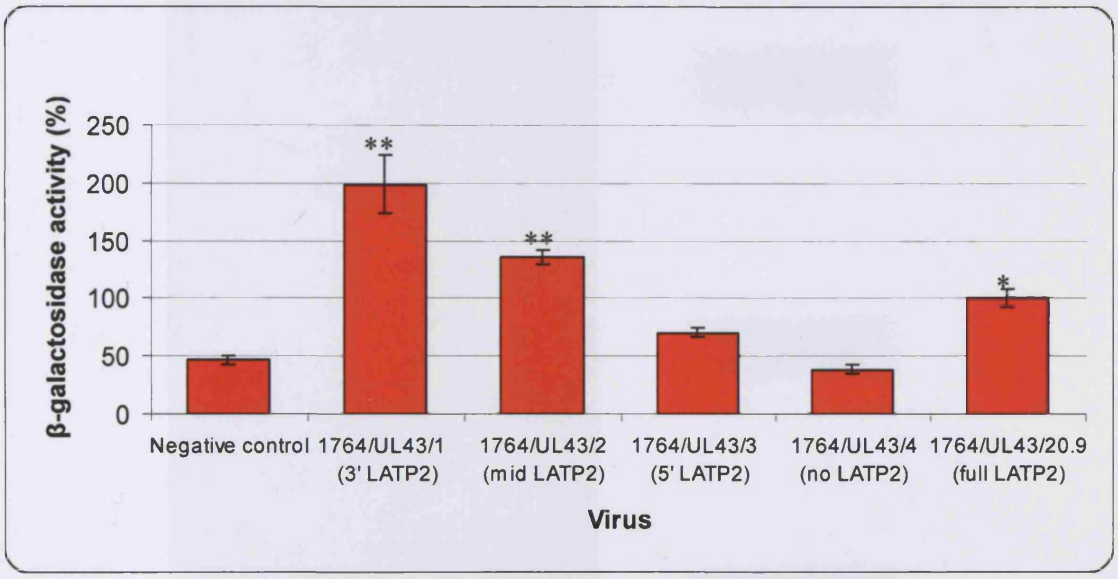
The levels of GFP were assessed by visualisation only. The fourth set of infections were examined after 48 hours for eGFP expression under a fluorescent microscope and photographed. Examples of GFP expression in the infected cells are shown in figure 3-15.

Figure 3-14 ***In vitro*** **β-galactosidase assays.**

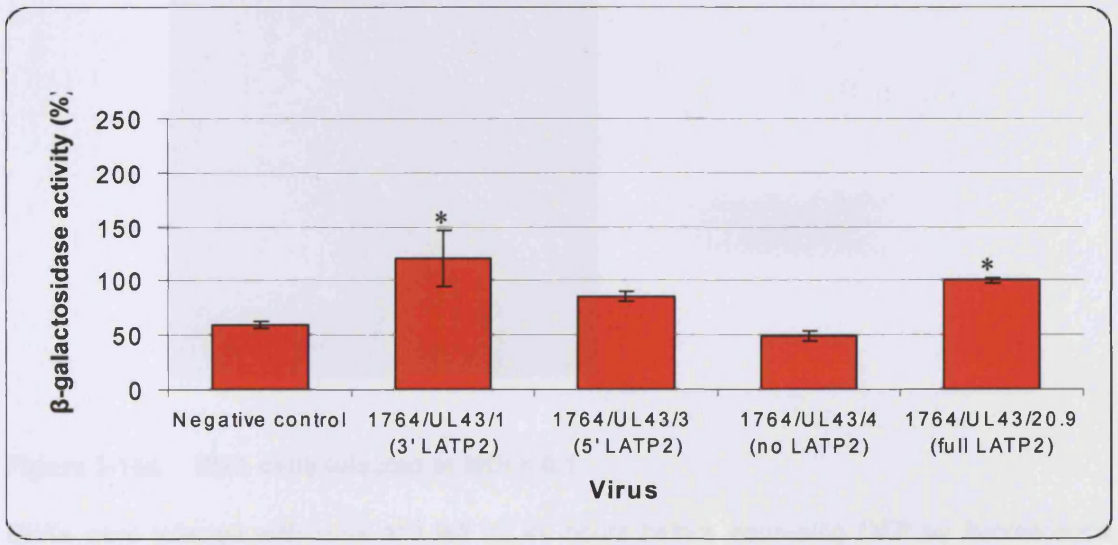
BHKs (a) and ND7s (b, c) were infected with virus at different MOIs and left for 48 hours before assay. (Graph c, no data for 1764/U_L43/2). Negative control = 1764 virus. (* - $p<0.01$, ** - $p<0.001$)



a. BHK – MOI = 0.1



b. ND7 – MOI = 1



c. ND7 – MOI = 5

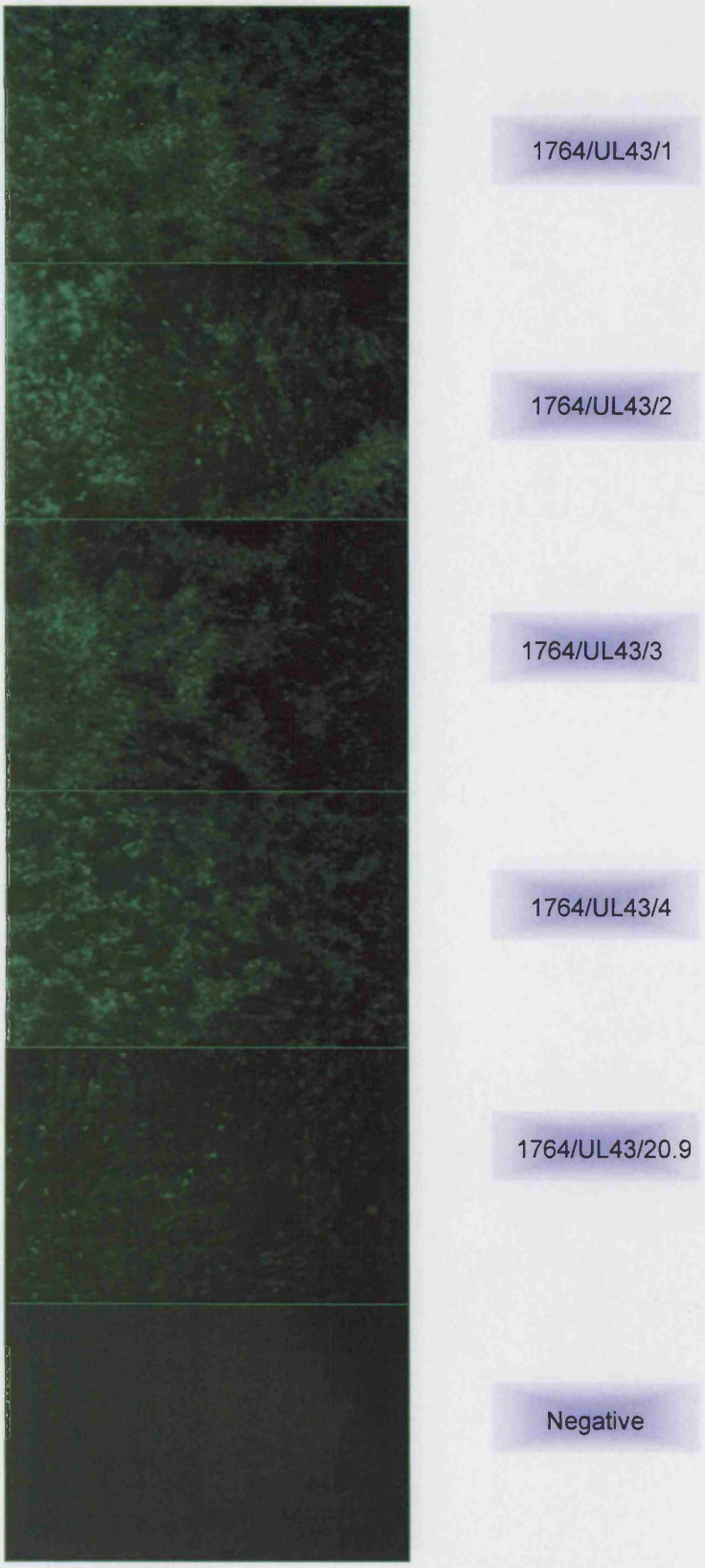


Figure 3-15a BHK cells infected at MOI = 0.1

BHKs were infected with virus and left for 48 hours before visualising GFP by fluorescence microscopy. Negative control is 1764 virus.

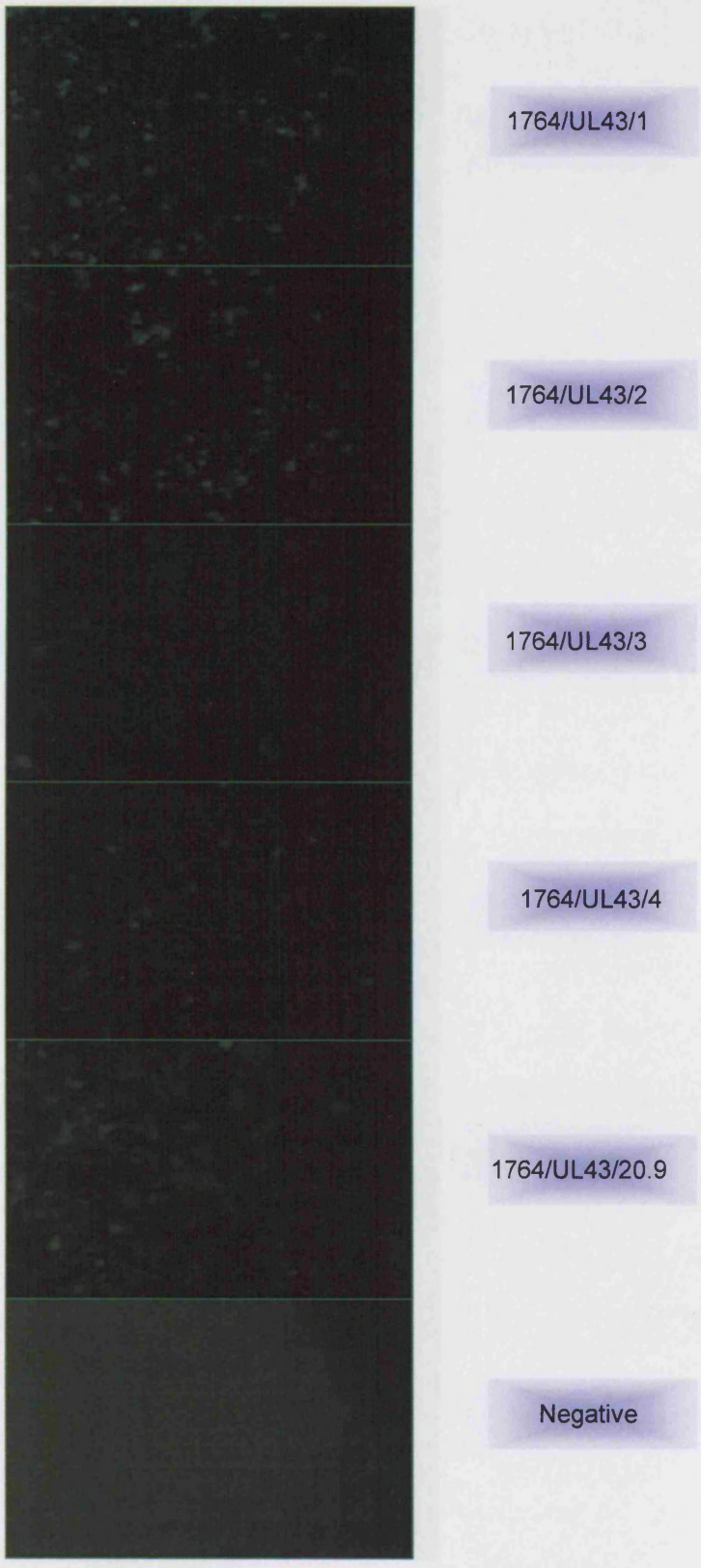


Figure 3-15b ND7 cells infected at MOI = 1

ND7s were infected with virus and left for 48 hours before visualising GFP by fluorescence microscopy.

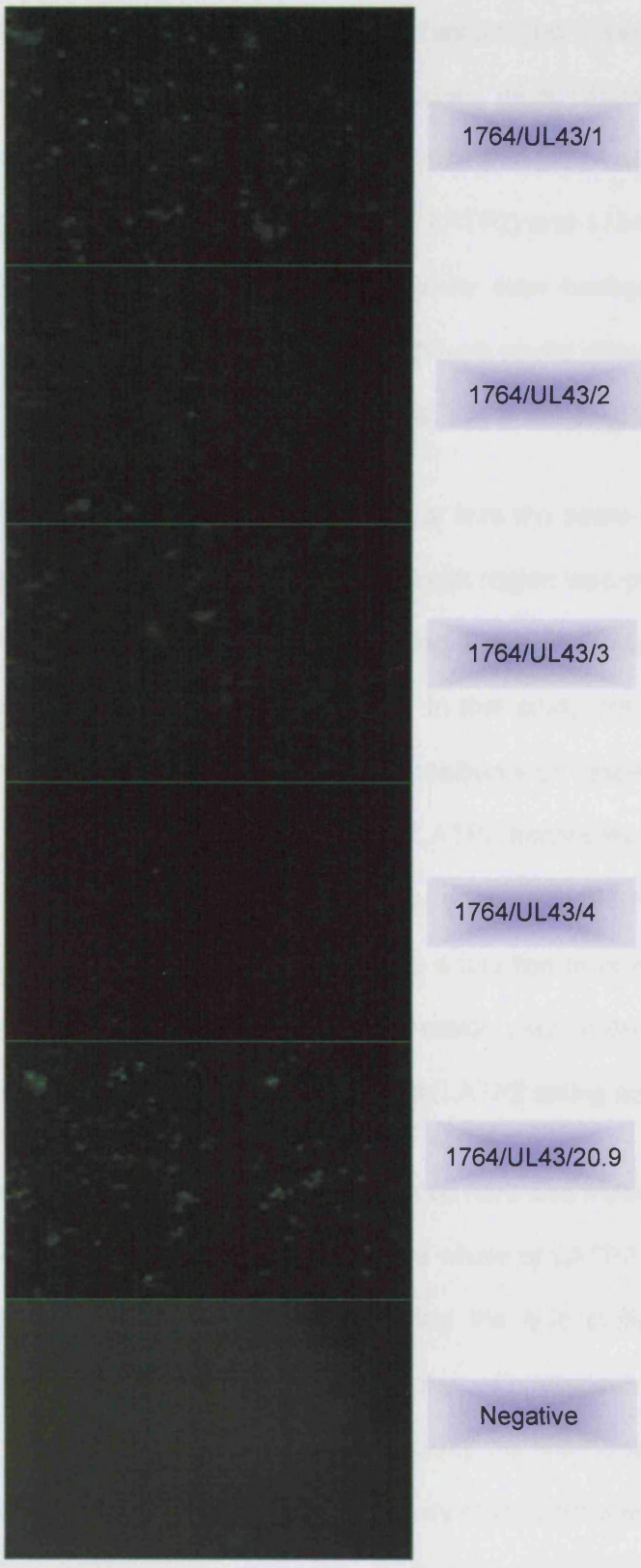


Figure 3-15c ND7 cells infected at MOI = 5

ND7s were infected with virus and left for 48 hours before visualising GFP by fluorescence microscopy.

These results show that the 1764/U_L43/20.9 virus has a higher level of *lacZ* expression than any of the other viruses ($p<0.001$) in BHK cells, all of which do not give more expression than background levels. However, in neuronal ND7 cells, at an MOI of 1, 1764/U_L43/1 (right half LATP2), 1764/U_L43/2 (mid LATP2) and 1764/U_L43/20.9 all give significantly higher ($p<0.001$ – $p<0.01$) *lacZ* activity than background levels. This appears to be true at the higher MOI of 5 also, although as the data for the mid LATP2 virus is missing due to loss of two sets of infections, this is not conclusive.

In a similar study, viruses with deletions of more or less the same sections of LATP2 gave the most expression of *lacZ* when the full-length region was present in BHK and ND7 cells, with the other deletion viruses giving 2.5 – 4 times less expression depending on cell type (Berthomme *et al.* 2001). In that study, the viruses contained LATP2 fragments in the natural orientation and position with respect to LAP1; which differs from the work described here, where the LATP2 fragments are in the reverse orientation and position with respect to the natural orientation of LAP1. Therefore the *lacZ* expression seen by the other group could be a function of promoter activity from LAP2 within the LATP2 region, whereas the expression seen in the results described above can only come from the LAP1 promoter, with LATP2 acting as an enhancer.

The fact that the two sets of results (those presented here and those of Berthomme *et al*) seem to concur in BHK cells, in both cases the whole of LATP2 being required for optimum transgene expression, shows that during the lytic cycle in BHK cells in culture, most activity comes from the LAP1 promoter, with elements across the whole of LATP2 being required for maximal enhancer activity. As mentioned, when LATP2 is in its natural position, promoter and enhancer activity could both affect *lacZ* expression in the vectors. Direct comparison of absolute *lacZ* expression levels between the two sets of viruses would need to be looked at in order to confirm this.

In neuronal ND7 cells, the results obtained differ. Berthomme *et al* found that the virus infections gave the same pattern of results as in BHK cells, however the data above differs in the two cell types. This could be due to the low *lacZ* expression levels from these viruses *in vitro*, as discussed previously, such that because they are below the detectable range of the assay in some viruses, the differences are just not being seen.

The use of cell lines may not be an ideal method to study the efficiency of transcriptional regulation in some cases. Cell lines may not represent the cellular environment *in vivo*, probably not containing the transcription factors present in the cell *in vivo* and may not therefore be physiologically relevant for the study of HSV latency.

3.7 *IN VIVO* TESTING OF RECOMBINANT VECTORS

To investigate the activity of LATP2 in the viruses described further, *in vivo* experiments were carried out. 1764/U_L43/20.9 has previously been shown to give higher levels of *lacZ* expression *in vivo* than that observed *in vitro* (Palmer *et al.* 2000), so it was thought likely that the LATP2 deletion viruses would show a similar expression pattern.

3.7.1 Testing in the PNS

The use of the footpad inoculation model for examination of expression from LAT promoters has been well documented (Cook and Stevens 1973b; Stevens and Cook 1971b) (see section 2.4.1). 3-week-old Balb/C or Balb/C-SCID mice were injected in the left footpad with 20μL of each virus at a titre of 1 x 10^8 pfu/mL (Total virus injected = 5 x 10^6 pfu). The dorsal root ganglia from the lumbar region ipsilateral to the injection site were removed after 3 days (Balb/C) and 1 month (Balb/C-SCID). These time-points correspond to acute infection and latent infection. DRG were post-fixed and

stained with X-Gal. *LacZ* positive neurons from DRG at positions L3-L6 were then counted.

Initially, 3 mice per virus, per time-point were inoculated and DRG processed. However, it was found that there was great variability in the results (data not shown) using this number of animals per group, so the experiments were repeated with 10 mice per time-point.

The method of measuring *lacZ* activity in whole ganglia has been investigated before in our laboratory. It has previously been attempted to measure *lacZ* expression from a virus with a 1764 backbone by chlorophenolred-ß-D-galactopyranoside (CPRG) assay. This was met without success even at lytic time-points due to the relatively low level of expression. Dobson *et al.* (Dobson *et al.* 1995) were able to do this at early time-points using a wild-type KOS-based virus, but this did not give enough expression during latency to allow quantification by CPRG assay. Dobson *et al* counted *lacZ* expressing-neurons at lytic times as well as measuring by CPRG and showed that the two results obtained tallied. Therefore it is reasonable to count *lacZ*-positive neurons in DRGs as a measure of promoter activity.

On examining the stained ganglia, it was observed that two populations of *lacZ* – positive neurons were present; darkly stained and lightly stained. Those that were darkly stained were often associated with stained axons highlighting the tracking of the virus from the footpad to the ganglia. The more lightly stained neurons often had a 'speckled' appearance as observed by others previously (Dobson *et al.* 1990; Ho and Mocarski 1989). The two different populations might correspond to unequal amounts of virus reaching the neurons of the ganglia from the footpad. Not all neurons will receive the same amount of virus during footpad injection. In order to improve the recording of *lacZ* expression and obtain a more meaningful, semi-quantitative result, a score was given to each neuron counted – 1 for a lightly stained neuron and 2 for a

darkly stained neuron, where there was obviously greater transgene expression. Results are therefore shown as a weighted *lacZ* 'score' to represent the expression level.

3.7.1.1 LATP2 activity 3 days post-injection

Results for 3 days post-injection (p.i.) are shown in table 3-2 and figure 3-16. Selected ganglia from each virus-set are shown in figure 3-17.

The results of the *lacZ* quantification show the effect of deletions of the LATP2 region on *lacZ* expression *in vivo* at 3 days p.i. Deleting either the 5' or 3' half of LATP2 (viruses 1764/U_L43/1 and 1764/U_L43/3) reduces *lacZ* expression significantly ($p<0.001$) compared to that from 1764/U_L43/4 (i.e. expression from just LAP1). Expression levels of *lacZ* from the virus 1764/U_L43/20.9 and the virus with the mid-section of LATP2 (1764/U_L43/2) are similar to that without LATP2 (1764/U_L43/4). There does not appear to be much enhancer activity at all at this time from LATP2. This would also suggest that there are repressive elements in both halves of the LATP2 promoter; the action of which are only underlined when the other half is not present. When neither, both, or fragments of both halves are present, the repression is not seen. Perhaps a mechanism exists in the region in which a mirroring effect occurs, active and repressive elements in each balancing out the activity of each half when both are present. The fact that the repression is not seen when the extreme ends of LATP2 are removed as in virus 1764/U_L43/2 perhaps points to the repressive elements being located in these regions.

Although the GFP expression was not quantified, examining the DRGs under fluorescent light shows the general level of this reporter gene activity at 3 days p.i. The most notable observation is that levels of *lacZ* and GFP activity are similar in the DRGs (N.B. Figure 3-17 only shows 1 DRG out of up to 40 extracted for each virus).

VIRUS	PALE NEURONS	DARK NEURONS	TOTAL SCORE	S.E.M.
1764/U_L43/1 (3' HALF LATP2)	27.8	24.6	77	7.34
1764/U_L43/2 (MID LATP2)	47	51.1	149.2	16.40
1764/U_L43/3 (5' HALF LATP2)	27.7	4.8	37.3	3.17
1764/U_L43/4 (NO LATP2)	42.6	49.5	141.6	14.93
1764/U_L43/20.9 (FULL LATP2)	44	60.7	165.4	10.65

Table 3-2 **Averaged counts of *lacZ*-positive neurons 3 days p.i.**
LacZ positive neurons were recorded per animal, and scored according to whether staining for *lacZ* was pale or dark (n=10).

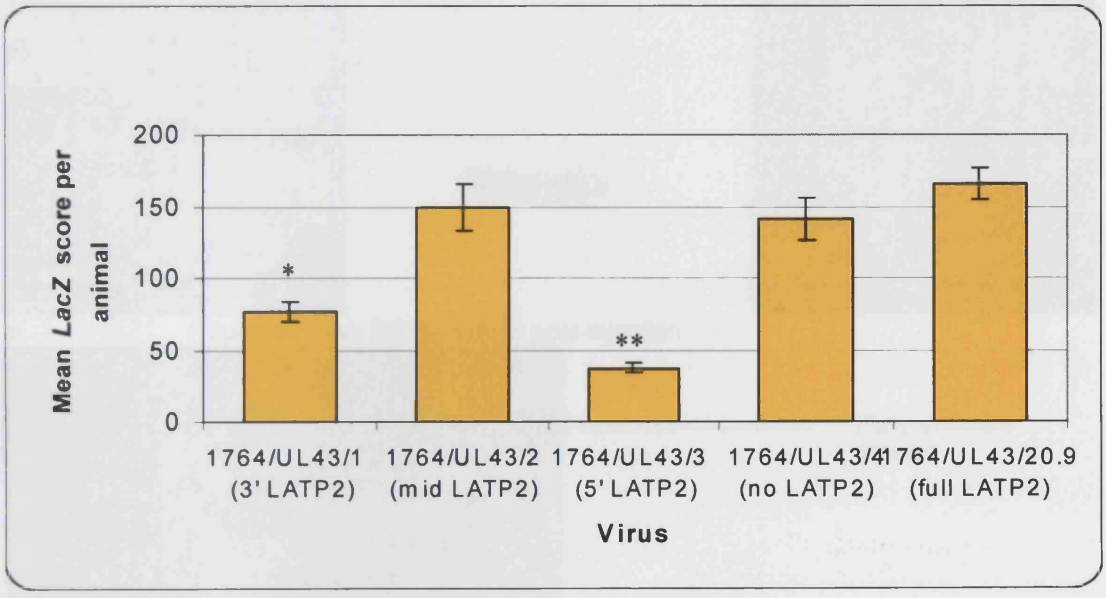
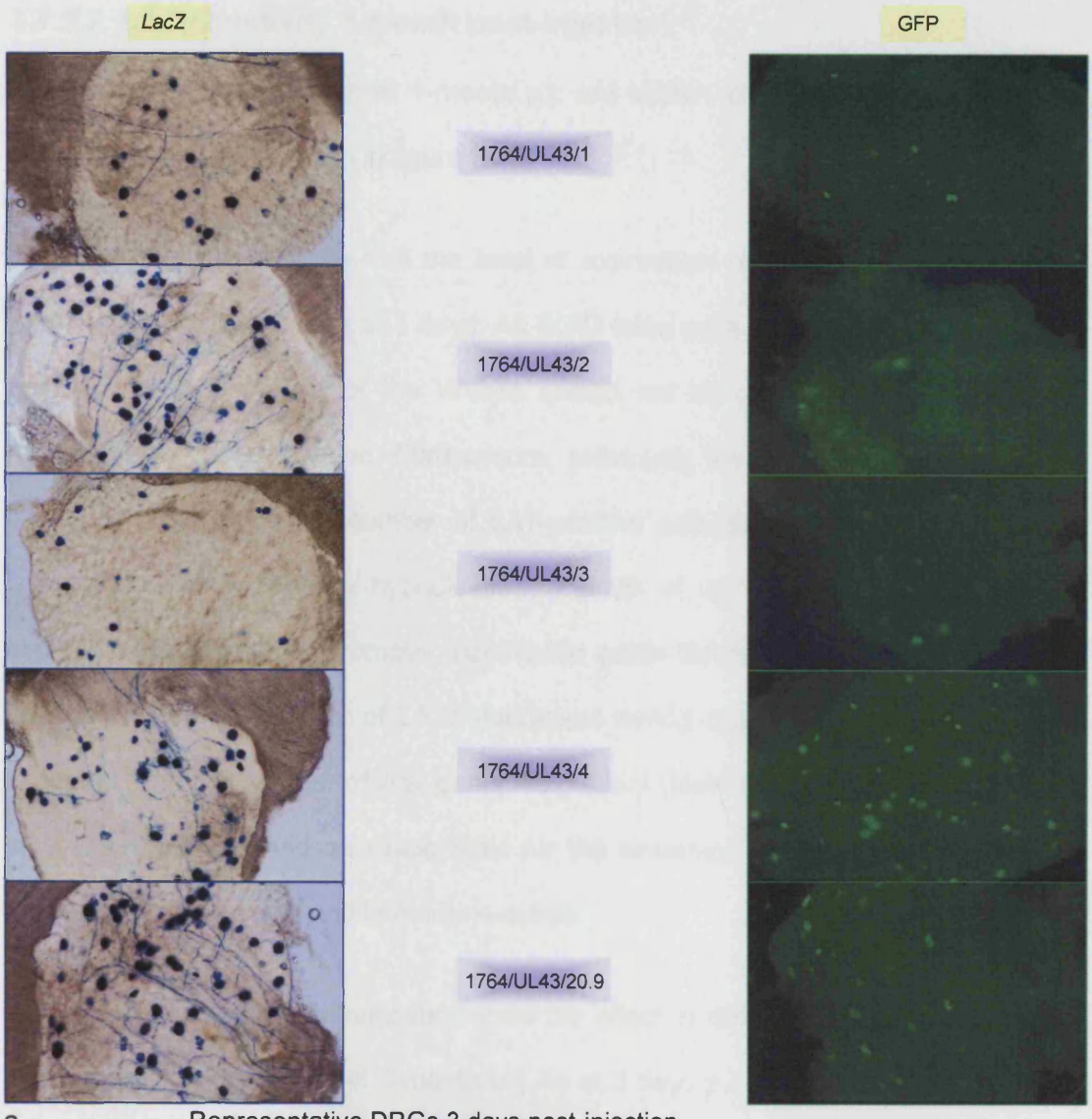
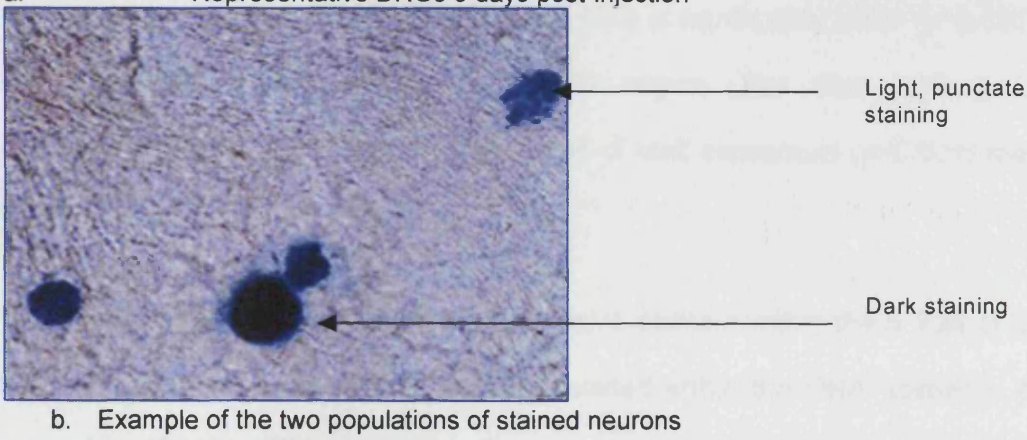


Figure 3-16 ***LacZ* expression from LATP2-deletion viruses 3 days post-injection in Balb/C mice**
Weighted *lacZ*-positive neuron scores give a semi-quantitative measure of *lacZ* expression from the LATP2 deletion viruses.
* - $p<0.01$, ** - $p<0.001$.



a. Representative DRGs 3 days post-injection



b. Example of the two populations of stained neurons

Figure 3-17 Photographs of *lacZ* and GFP expression from LATP2-deletion viruses 3 days post-injection in Balb/C mice

a. Selected examples of ganglia from animals 3 days post-injection showing GFP and *lacZ* expression.

b. Magnified DRG showing the two types of staining seen: paler, punctate staining and darker staining.

3.7.1.2 LATP2 activity 1 month post-injection

Results for *lacZ* expression at 1-month p.i. are shown in table 3-3 and figure 3-18. Selected ganglia are shown in figure 3-19.

These results demonstrate that the level of expression of *lacZ* at 1 month is much lower (up to 12 times) than at 3 days. As SCID mice were used for the 1-month time-point, immune clearance of the viruses should not be playing a large part in the reduction in *lacZ* expression. Furthermore, previously the 1764/U_L43/20.9 virus was shown to have the same number of LAT-positive cells as *lacZ*-positive cells during latency *in vivo* by *in situ* hybridisation (Palmer *et al.* 2000). Thus the reduced expression is not due to promoter inactivation within the cassette. It has been shown previously that the number of LATs expressed during latency is less than would be expected from the number of viral genomes present (Mehta *et al.* 1995; Ramakrishnan *et al.* 1994). This could be responsible for the observed reduction in expression of transgene between lytic and latent time-points.

The results of the *lacZ* quantification show the effect of deletions of the LATP2 region on *lacZ* expression *in vivo* at 1-month p.i. As at 3 days p.i., expression from the virus containing only the 5' half of LATP2 (1764/U_L43/3) is significantly lower ($p<0.001$) than that from the virus containing no LATP2 region. The virus without LATP2 (1764/U_L43/4) gives significantly higher levels of *lacZ* expression ($p<0.001$) than that with the full LATP2.

These results suggest that there is a repressive element within the 5' half of LATP2 acting at latent time-points. It is probably located within the DNA upstream of that present in 1764/U_L43/2 (the mid section), as this virus gives a similar level of *lacZ* expression to that of the virus containing no LATP2. As the full LATP2 also contains this potentially repressive region and yet allows almost as much expression as the mid LATP2, there must be other elements also present to balance this repression.

The low expression level of *lacZ* from the virus containing the 5' half of LATP2 is interesting as this result does not concur with that seen by (Berthomme *et al.* 2001). This region alone in their virus was sufficient to give as much long-term expression as the whole region. The disparity between the two results can probably be explained by the difference in arrangement, orientation and location of LAT promoters/enhancer within the virus. The long-term activity seen from LATP2 when in the same orientation as LAP1 in that study appears to be abrogated when in the reverse direction as seen here.

Perhaps the most surprising result is that of *lacZ* expression levels from the virus without any LATP2 (1764/U_L43/4). Others have found that expression from LAP1 is very low or non-existent at latent time-points without some part of the LATP2 region present (Berthomme *et al.* 2000; 2001; Coffin *et al.* 1996b; Lokensgard *et al.* 1994; Lokensgard *et al.* 1997; Margolis *et al.* 1993). This must be contributed to the presence of the MMLV LTR in 1764/U_L43/4. As previously mentioned (section 1.1.3), the MMLV LTR has been shown to be capable of long-term expression of a transgene when linked to LAP1 (Carpenter and Stevens 1996; Dobson *et al.* 1990; Lokensgard *et al.* 1994). In the work presented here, the MMLV LTR is in the reverse orientation to LAP1, acting as an enhancer.

The presence of LATP2 in addition to the MMLV LTR has no advantage in these vectors; indeed it appears to be causing repression of this activity by MMLV LTR in varying degrees depending on which part is present. Thus the effects of the MMLV LTR promoter may be obscuring those of LATP2 on LAP1.

In figure 3-18, the examples of DRGs expressing GFP demonstrate that the same virus, without LATP2, that has the most *lacZ* expression also has the most GFP expression during latency, suggesting that any effect of the MMLV LTR on transgene expression is bi-directional, with promoter and enhancer activity.

VIRUS	PALE NEURONS	DARK NEURONS	TOTAL SCORE	S.E.M.
1764/U_L43/1 (3' HALF LATP2)	15.9	0.3	16.5	1.86
1764/U_L43/2 (MID LATP2)	22.7	1	24.7	3.07
1764/U_L43/3 (5' HALF LATP2)	2.5	0.1	2.7	0.60
1764/U_L43/4 (NO LATP2)	25.9	2.8	31.5	2.75
1764/U_L43/20.9 (FULL LATP2)	11.3	1.6	14.5	3.40

Table 3-3 **Averaged counts of *lacZ*-positive neurons 1 month p.i.**
LacZ positive neurons were recorded per animal, and scored according to whether staining for *lacZ* was pale or dark (n=10).

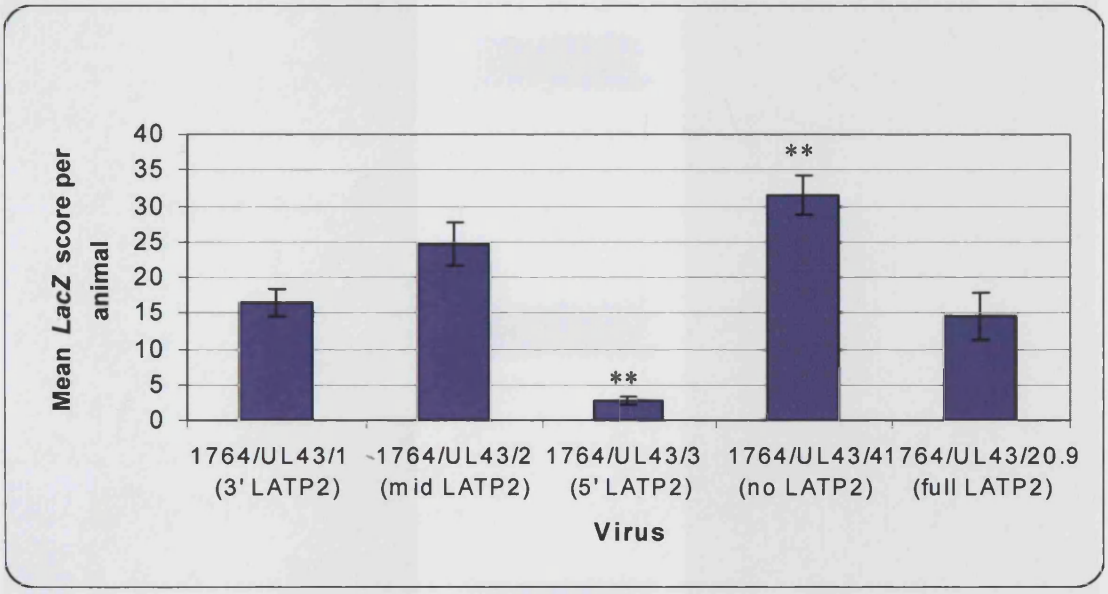


Figure 3-18 ***LacZ* expression from LATP2-deletion viruses 1 month post-injection in Balb/C SCID mice**
Weighted *lacZ*-positive neuron scores give a semi-quantitative measure of *lacZ* expression from the LATP2 deletion viruses.

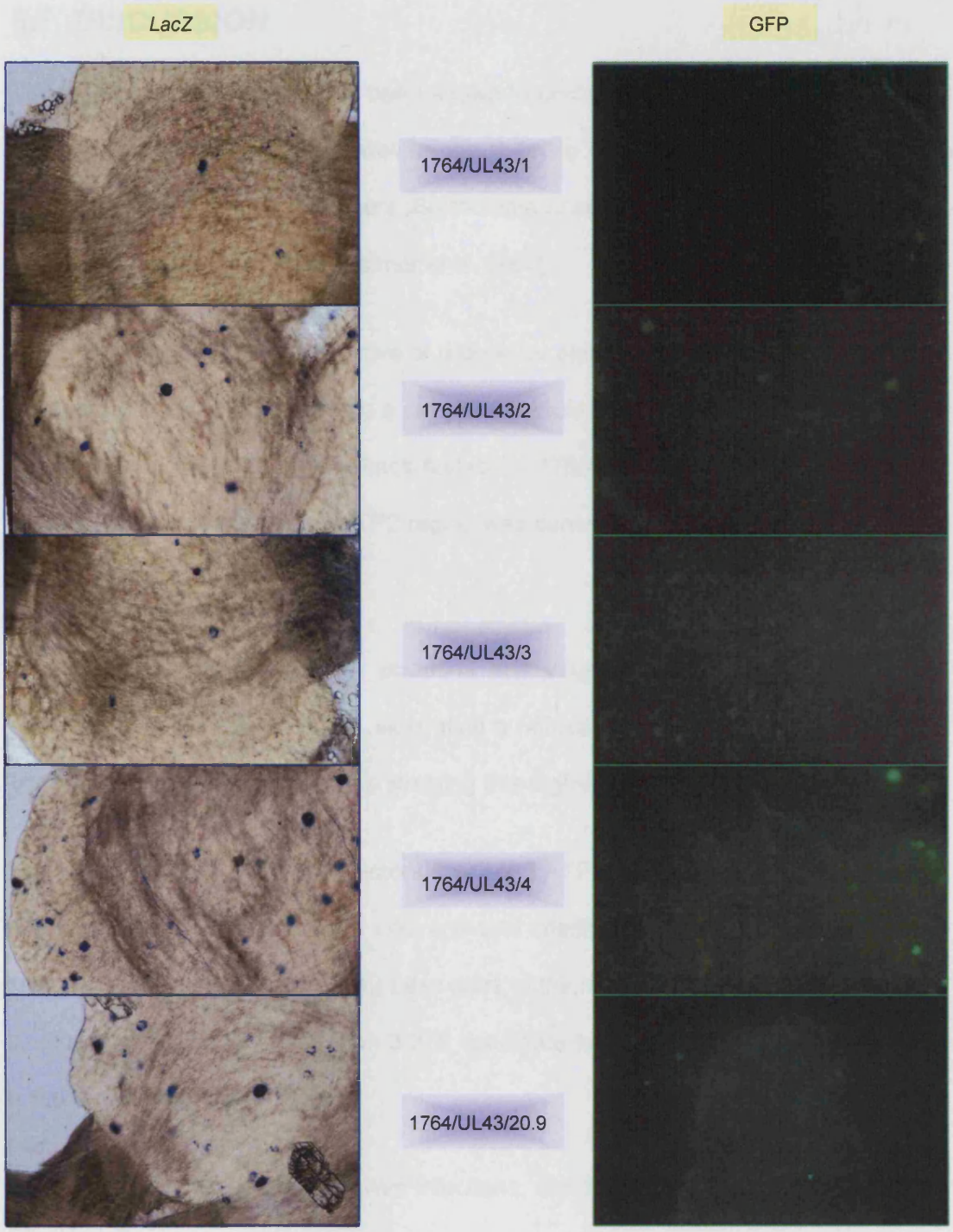


Figure 3-19 **Representative ganglia from Balb/C SCID mice 1 month post-injection showing *lacZ* and GFP expression.**

3.7 DISCUSSION

The LATP2 region of HSV1 has been shown to contain promoter, enhancer and long-term expression capabilities as well as the ability to confer long-term expression onto neighbouring exogenous promoters (Berthomme *et al.* 2000; Lachmann and Efstathiou 1997; Lokensgard *et al.* 1997; Palmer *et al.* 2000).

This chapter aimed to identify active or repressive elements of the LATP2 region within the context of a vector containing a cassette wherein LATP2 confers long-term activity on two promoters in a back-to-back fashion – 1764/U_L43/20.9 (Palmer *et al.* 2000). Deletion mutagenesis of the LATP2 region was carried out to create vectors based on 1764/U_L43/20.9.

The expression profiles of the plasmids and viruses appeared to be consistent, irrespective of the cells infected, illustrating a non cell-type specific enhancer activity. This was also reported by a group studying this region (Berthomme *et al.* 2000).

The results show that in the vectors studied, LATP2 contains repressive elements, which in plasmid transfections *in vitro* and viral infections *in vivo*, both lytic and latent, seem to be located in the first 242 base pairs of the region. Looking at the putative TF binding sites for LATP2 in section 3.2, a candidate for causing repression of the virus is the transcription factor MZF1.

In plasmid transfections and *in vivo* infections, the 3' half of LATP2 also appears to contain a repressive element, potentially within the last 720 base pairs. Potential transcriptional repressors of this region are MZF1 again and also p300, which can act as a repressor or activator depending on the environment. Of course these TFs are not necessarily present in the cellular environment in which these viruses were tested and further work beyond the scope of this thesis would be required to investigate the relevance of this.

The plasmid and virus with LATP2 completely deleted consistently gave similar or higher transgene expression than the plasmid and virus containing the full LATP2 region, in all situations studied except for lytic infections *in vitro*. The *in vitro* assays were not very conclusive however, due to the low expression afforded from most of the viruses when used to infect BHK cells and the low infection achieved in the ND7 cells. Differences in LAT promoter expression in different cell lines *in vitro* and *in vivo* has been observed before (Dobson *et al.* 1995). This group made deletions of LAP1 and commented that the differences seen in activity from the different constructs and viruses in different situations was probably due to the fact that the kinds and quantities of transcription factors present would vary depending on the cellular environment.

A possible reason that in some situations tested, the highest *lacZ* expression levels were seen when LATP2 was removed is that this configuration places the MMLV LTR closer to LAP1 than in any other plasmid/virus. The MMLV LTR is an enhancer and often increasing the proximity to the promoter results in increased promoter activity.

The presence of the MMLV LTR in these vectors obviously has an effect on *lacZ* expression from LAP1 and appears to be repressed by the presence of certain elements within LATP2. In light of this, the vector 1764/U_L43/20.9 could perhaps be improved for long-term expression of two transgenes by actually deleting LATP2 completely, as the MMLV LTR has its own enhancer and ability to confer long-term expression on exogenous promoters (Carpenter and Stevens 1996; Dobson *et al.* 1990; Laimins *et al.* 1984; Lokensgard *et al.* 1994) and the presence of both appears to be unnecessary.

However, as this thesis is focussed on characterising LATP2, it is clear that the presence of the MMLV LTR in these vectors is not ideal. As the MMLV LTR contains an enhancer itself, it is obscuring the effects of the enhancer activity of LATP2, making it difficult to assess purely the effects of LATP2 on transgene expression.

CHAPTER 4:

CONSTRUCTION AND TESTING OF HSV1 VECTORS CONTAINING LATP2 DELETION CONSTRUCTS (II)

4.1 INTRODUCTION

In Chapter 3, it was shown that LATP2 deletion viruses based on 1764/U_L43/20.9 gave varying levels of transgene expression. The presence of the MMLV LTR promoter/enhancer in the viruses also conferred activity onto the LAP1 promoter and confused the results. To assess the effect of LATP2 alone on expression from LAP1, a second set of LATP2 deletion viruses was created, without the MMLV LTR and GFP region of the expression cassette.

To characterise the LATP2 region further, it was thought appropriate to also create a third set of LATP2 deletion viruses, in which LATP2 is placed in the same orientation as LAP1. This should enable the directionality of LATP2 to be studied, which is relevant to the starting vector, 1764/U_L43/20.9, in which LATP2 is exerting activity in both directions and in which it was not possible to quantify the activity of LATP2 on GFP expression due to the presence of the MMLV LTR (chapter 3).

It is known that the ICP4 protein negatively regulates the LAP2 promoter contained within LATP2 *in vitro* (Goins *et al.* 1994). The repression was shown not to be due to the direct binding of ICP4 to LAP2, as is the case with LAP1 (Batchelor *et al.* 1994), as LAP2 does not contain an obvious binding site for the protein. It was suggested that the repression might occur via the interaction of ICP4 with pre-initiation complex transcription factors. In order to investigate this further and see whether it affected enhancer activity of LATP2, the region identified by Goins *et al.* (Goins *et al.* 1994) as being repressed by ICP4 in LAP2, was removed from the 5' half of LATP2 (equating to LAP2) and the full LATP2. These LATP2 deletion fragments were then used to create a further four viruses, the fragments being placed in both orientations with respect to LAP1 within the expression cassette.

4.2 LATP2 DELETION PLASMIDS

4.2.1 Series 2 plasmids– LATP2 in the opposite orientation to LAP1

A series of seven LATP2 deletion plasmids were created based on those described in the previous chapter, but lacking the MMLV LTR and GFP. The same deletion fragments of LATP2 were used, as well as two additional ones, the 5' half minus the 'ICP4 responsive region' (these are referred to as ΔICP4 throughout the chapter) and the full LATP2 minus the 'ICP4 responsive region'. Details of construction are provided below.

4.2.1.1 Method of construction

pR20.9 was digested with *Kpn1/Nsi1* to remove the LAP1/LATP2/MMLV/GFP fragment. LAP1 was digested from pP1*lacZ*Srf with *Kpn1/Nsi1* and religated into the digested pR20.9 plasmid.

An oligonucleotide was then ligated after LAP1, to allow reinsertion of LATP2. This was the same as that described in section 3.2.1.

The LATP2 deletion fragments were all digested from pP2BSK (M. Robinson) as follows: -

(1) *PpuM1 – Spe1* (HSV nt 119502 - 120219)

(2) *BspE1 – Bbs1* (HSV nt 119107 – 119677)

(3) *Xho1 – PpuM1* (HSV nt 118866 – 119502)

(4) No LATP2

(5) *Xho1 – Spe1* (HSV nt 118866 – 120219)

The ΔICP4 LATP2 fragments were created by removing nucleotides 119420 – 119502 as follows: The 5' half of LATP2 was digested with *TaqI*/T4 pol to remove the last 82bp. The full LATP2 had the ICP4 region removed by PCR from each half of LATP2 (excluding the ΔICP4 region). The following primers were used in a standard PCR reaction of 30 cycles:

(3' half) Primer 'FOR': 5' GAATTCGGACCCAAACGACAGGGGGCG

(5' half) Primer 'REV': 5' CTTAAGACGAGTGGGATAACGGG

The product of primer 'FOR' was cut with *Xba1* and inserted into pBluescript. The product of primer 'REV' was cut with *Xho1* and inserted into the plasmid above by digesting the pBluescript + 3' half LATP2 fragment with *EcoR1/T4/Xho1* and then ligating the two. This removed the same 82bp section of DNA from the centre of LATP2. The final cloned product structure was confirmed by sequencing.

These deletion fragments were then ligated into the cassette at the oligonucleotide by digestion with appropriate enzymes to give the series of LATP2 deletion plasmids (Figure 4-1).

4.2.2 Series 3 plasmids – LATP2 in the same orientation as LAP1

A third series of six LATP2 deletion plasmids were created, using the identical fragments to those in series 2, but inserted into the cassette in the opposite orientation, such that the LATP2 fragments were in the same orientation as LAP1. The plasmid without LATP2 is the same for both series (p4a). The series of plasmids created is shown in figure 4-1.

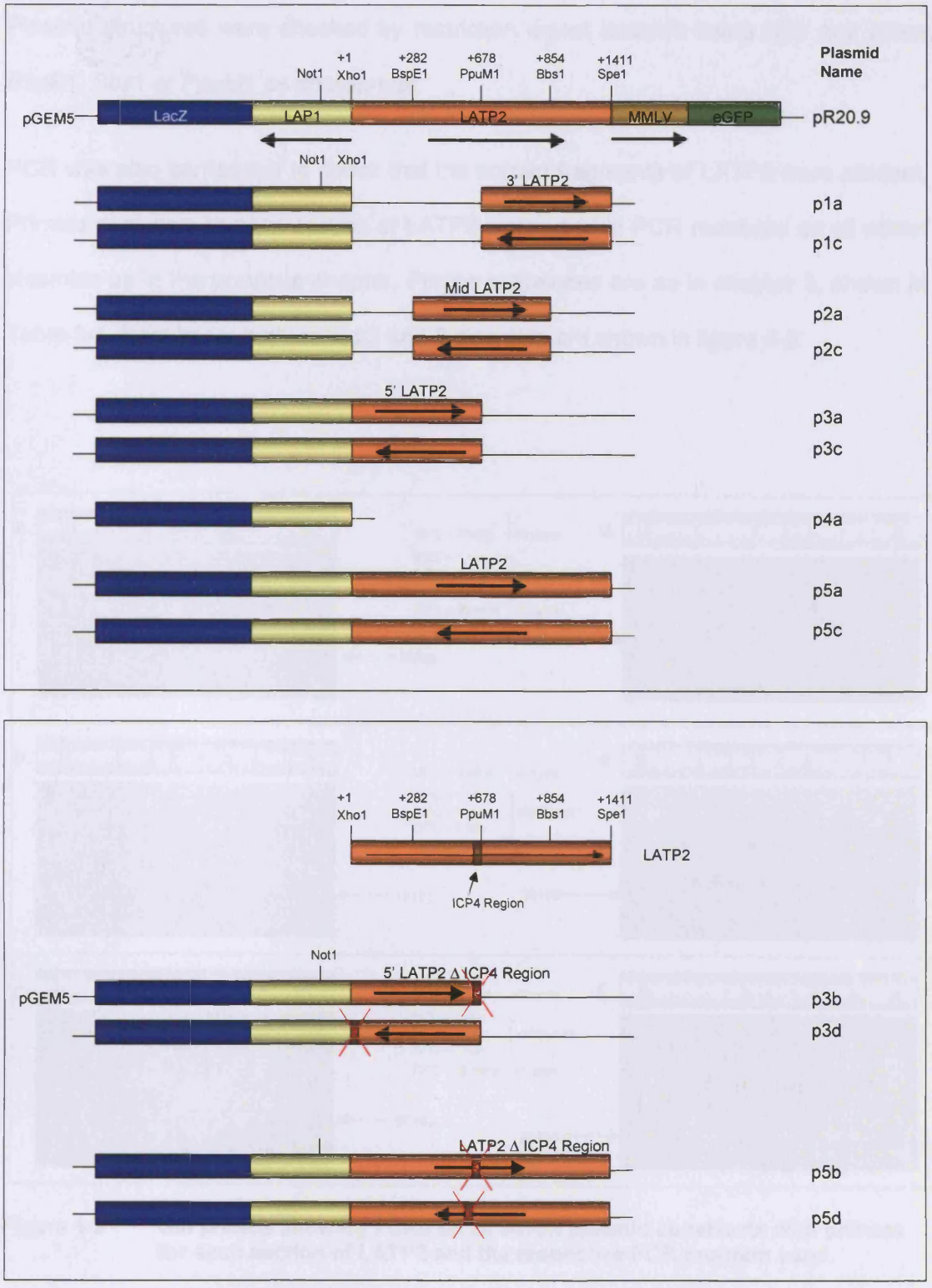


Figure 4-1 Schematic representation of LATP2 deletion cassettes

Restriction sites used to clone LATP2 fragments from pP2BSK are shown along with numbering relative to the start of LATP2 in the same plasmid. The brown block indicates the ICP4 responsive region removed. Both series of deletion cassettes are shown, the arrows denoting the orientation of LATP2, LAP1 and MMLV.

4.2.3 Analysis of plasmids

Plasmid structures were checked by restriction digest analysis using *NotI* and either *BspE*1, *Bbs*1 or *PpuM*1 as appropriate.

PCR was also carried out to check that the correct fragments of LATP2 were present. Primers exclusive to each section of LATP2 were used in PCR reactions on all seven plasmids as in the previous chapter. Primer sequences are as in chapter 3; shown in Table 3-1. Results for both series 2 and 3 plasmids are shown in figure 4-2.

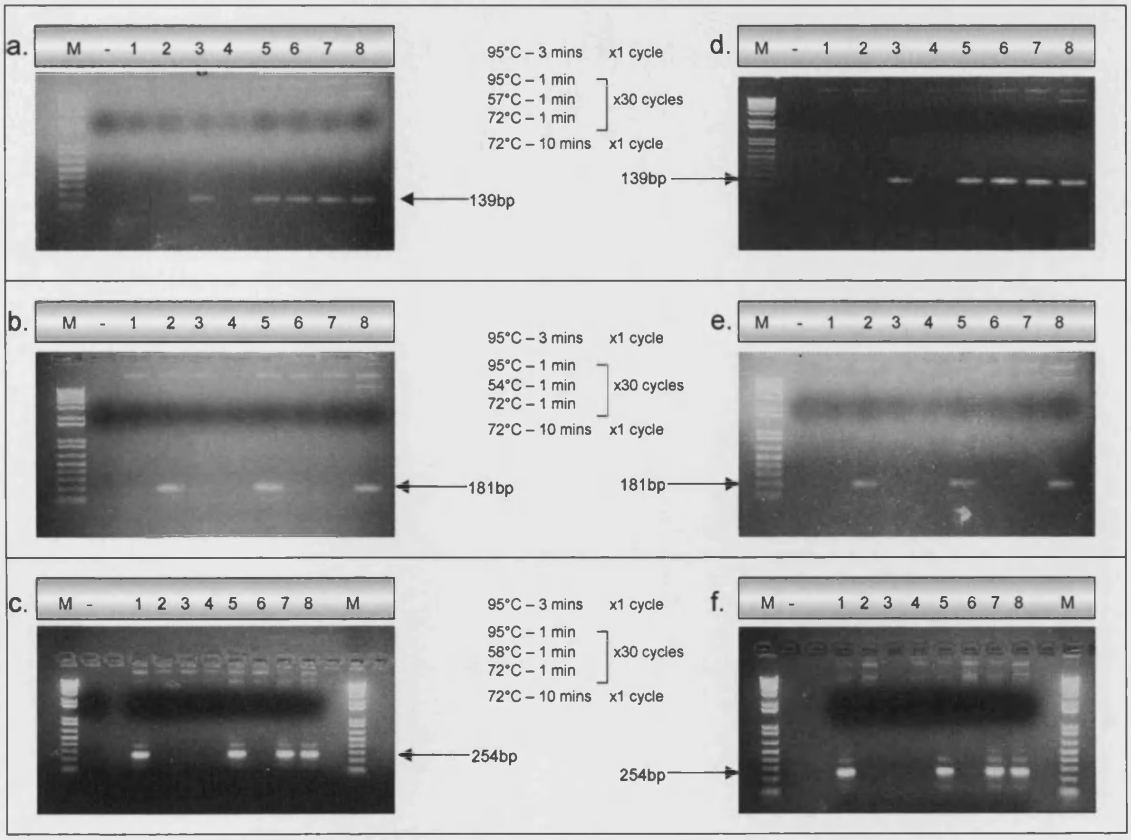


Figure 4-2 **Gel photos showing PCRs on all seven plasmid constructs with primers for each section of LATP2 and the respective PCR program used.**

a & d Primers for 5' half LATP2. b & e Primers for mid LATP2.
c & f Primers for 3' half LATP2.
a b & c Series 2 Plasmids M = Marker, 1 = p1a, 2 = p2a, 3 = p3a, 4 = p4a, 5 = p5a, 6 = p3b, 7 = p5b, 8 = pR20.9.
d e & f. Series 3 Plasmids M = Marker, 1 = p1c, 2 = p2c, 3 = p3c, 4 = p4a, 5 = p5c, 6 = p3d, 7 = p5d, 8 = pR20.9

The cassettes were then excised from the pGEM5 (Promega) backbone with *Srf*1 and ligated into the plasmid p35mod at the unique site *Nsi*1 as in section 3.2.1.

Because the insertion into p35mod can occur in either orientation due to the double-blunt digestion and ligation necessary, restriction digests of the resulting plasmids were carried out firstly to check that the insertion had taken place and secondly to identify which orientation each cassette was in relative to U_L43. Figure 4-3, table 4-1 and figure 4-4 show the structure of the cassettes in U_L43 and the restriction digests by *Not*1.

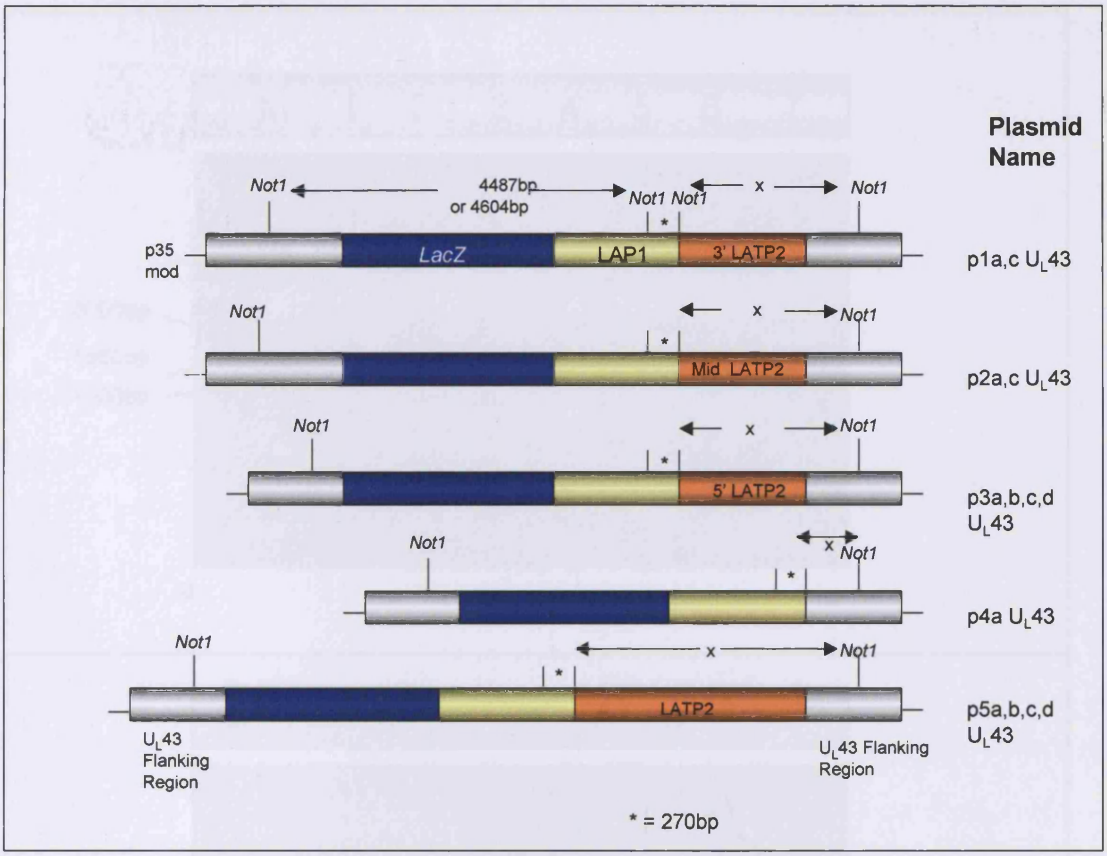


Figure 4-3 **Schematic representation of the cassettes in the UL43 flanking regions**

NB. The distance between the cassette and the first *Not1* site in U_L43 is either 315bp or 432bp on either side of the cassette, depending on orientation of the cassette within U_L43.

Plasmid Name	Size of x in orientation A	Size of x in orientation B
p1a,c	1448	1565
p2a,c	1287	1404
p3a,c	1393	1510
p4a	715	832
p5a,c	2126	2243
p3b,d	1205	1322
p5b/d	2044	2161

Table 4-1 **Difference in size of fragment 'x' depending on the orientation of each cassette.**

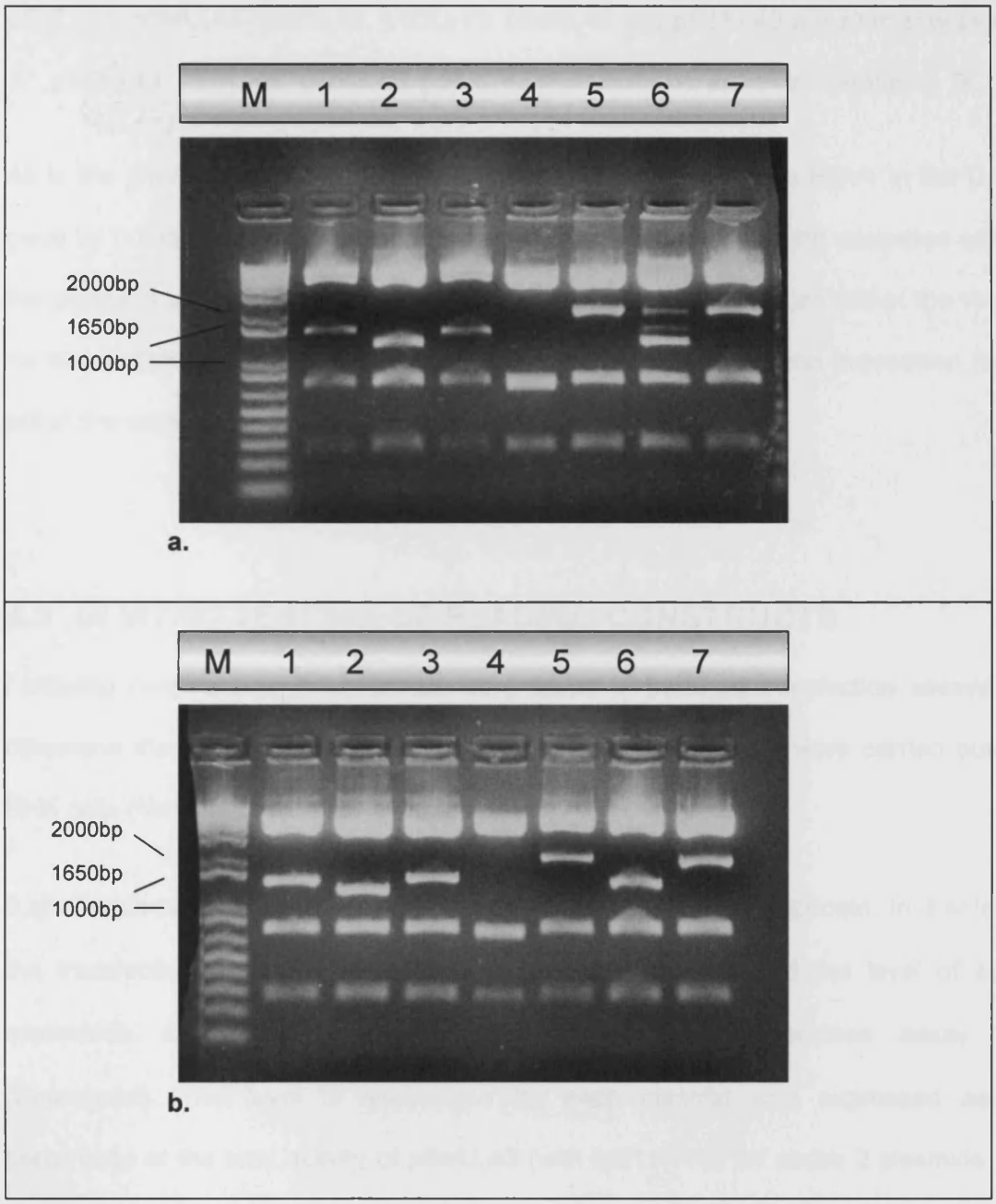


Figure 4-4 **Digestion with *Not1* of the cassettes in UL43 flanking regions.**

a. Gel photo of digests of series 2 plasmids with *Not1*.
M = 1kb+ ladder; 1 = p1a/U_L43; 2 = p2a/U_L43; 3 = p3a/U_L43; 4 = p4a/U_L43; 5 = p5a/U_L43; 6 = p3b/U_L43; 7 = p5b/U_L43.

b. Gel photo of digests of series 3 plasmids with *Not1*.
M = 1kb+ ladder; 1 = p1c/U_L43; 2 = p2c/U_L43; 3 = p3c/U_L43; 4 = p4a/U_L43; 5 = p5c/U_L43; 6 = p3d/U_L43; 7 = p5d/U_L43.

From Figures 4-3 and 4-4, it can be seen that the plasmids have ligated into the U_L43 flanking region plasmid (p35mod) in different orientations. p2a/U_L43, p4a/U_L43,

p5a/U_L43, p3b/U_L43, p5b/U_L43, p1c/U_L43, p5c/U_L43 and p5d/U_L43 are all in orientation 'A'. p1a/U_L43, p3a/U_L43, p2c/U_L43, p3c/U_L43 and p5d/U_L43 are all in orientation 'B'.

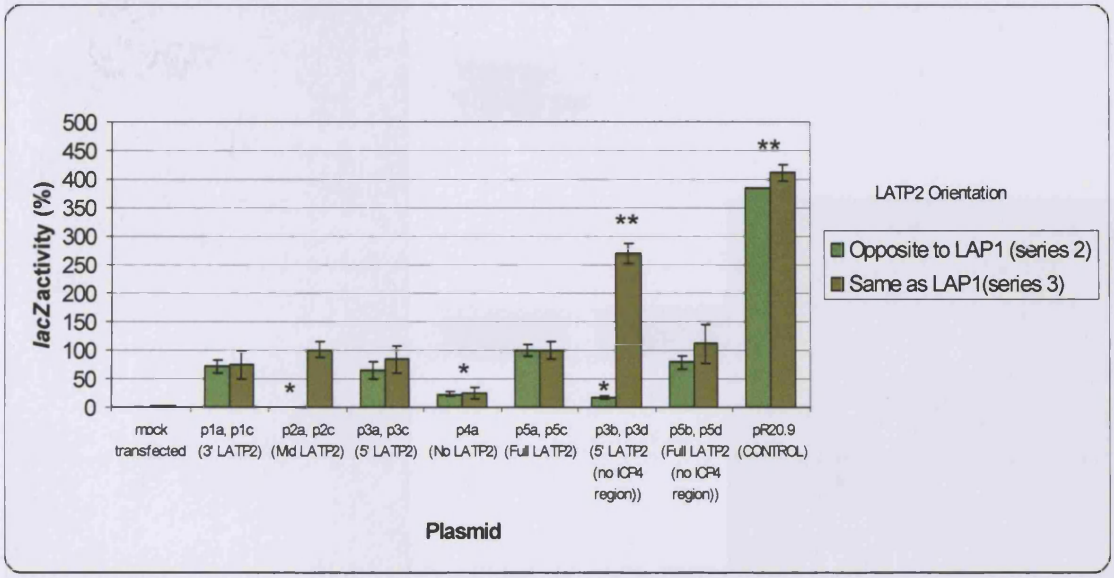
As in the previous chapter, the cassettes were to be inserted into HSV1 in the U_L43 gene by homologous recombination. The different orientations of the cassettes within the plasmids would also result in them being in differing orientations within the virus. As stated before, it was not thought that this would affect the gene expression from within the cassettes.

4.3 *IN VITRO* TESTING OF PLASMID CONSTRUCTS

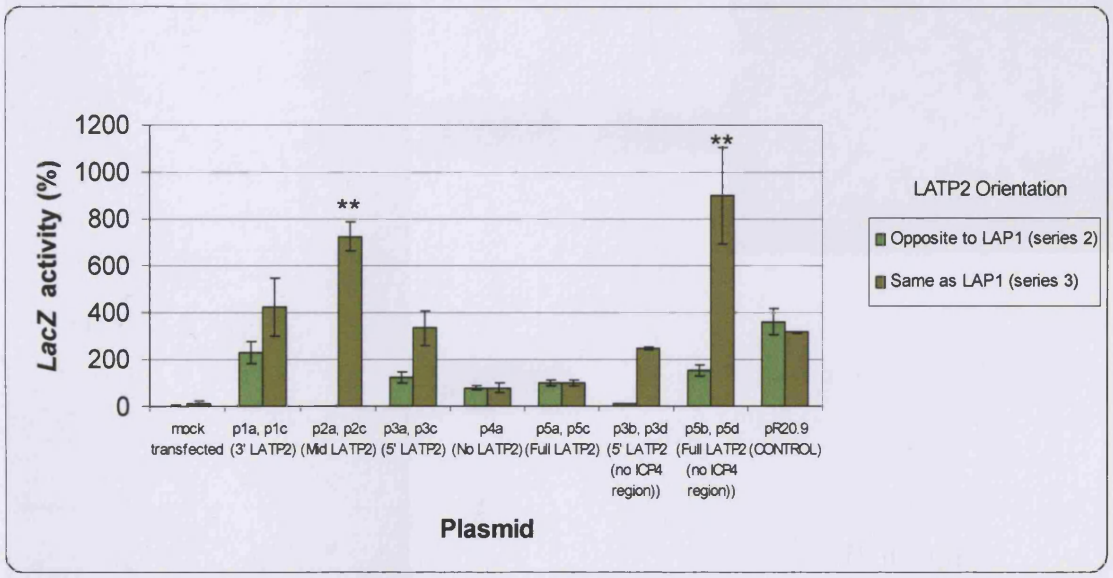
Following construction, the plasmids were tested in transient transfection assays to determine the levels of expression of the marker genes. These were carried out in BHK cells (fibroblast) and ND7 cells (neuronal).

5μg of each plasmid was transfected into each cell line, in quadruplicate. In 3 sets of the transfections, the cells were harvested after 48 hours and the level of *lacZ* expression determined using the high-sensitivity β-Galactosidase assay kit (Stratagene). The level of expression for each plasmid was expressed as a percentage of the total activity of p5a/U_L43 (with full LATP2) for series 2 plasmids, or p5c/U_L43 (again with full LATP2) for series 3 plasmids. The results are shown in figure 4-5.

The fourth set of transfections was examined after 48 hours, through staining with X-Gal in order to visualise *lacZ* expression. Examples of *lacZ* expression in the transfected cells are shown in figure 4-6a-d.



a. BHK Cells



b. ND7 Cells

Figure 4-5 ***LacZ* activity of series 2 & 3 LATP2 deletion plasmids *in vitro*.**

5μg plasmid was transfected into BHK cells (a) and ND7 cells (b). Data is represented as the % *lacZ* activity compared to that given by p5a/U_L43 (series 2) or p5c/U_L43 (series 3). The graphs depict average values of 3 transfections and the error bars are shown as SEM. (* - $p<0.01$, ** - $p<0.001$).
NB. p4a and pR20.9 are the same for both sets of plasmids, i.e. the directionality in the legend does not apply.

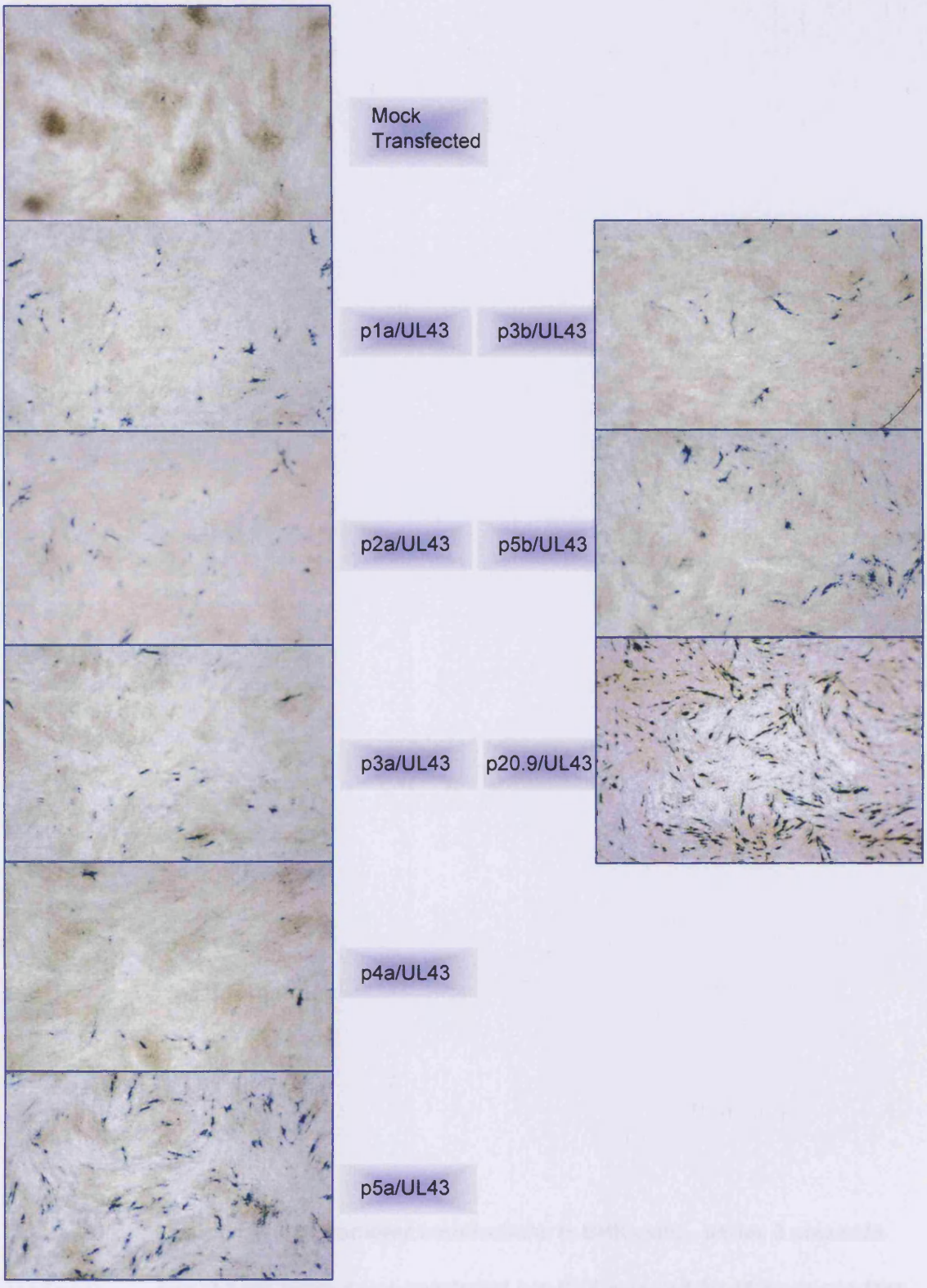


Figure 4-6a Photographs of transient transfections in BHK cells – series 2 plasmids

5μg of each plasmid was transfected into BHK cells, left for 48 hours and then stained with X-Gal to visualise *lacZ* expression.

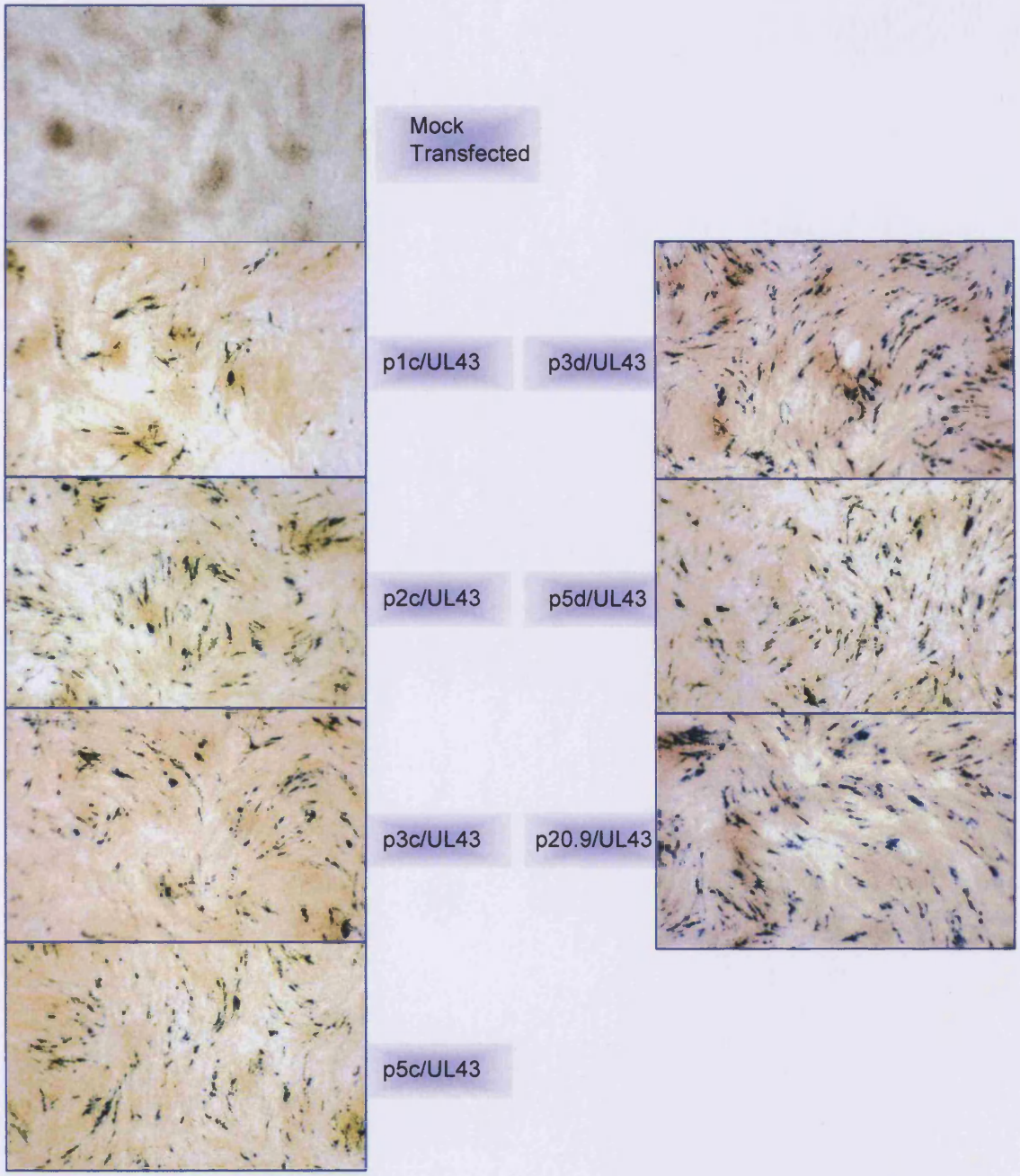


Figure 4-6b Photographs of transient transfections in BHK cells – series 3 plasmids

5μg of each plasmid was transfected into BHK cells, left for 48 hours and then stained with X-Gal to visualise *lacZ* expression.

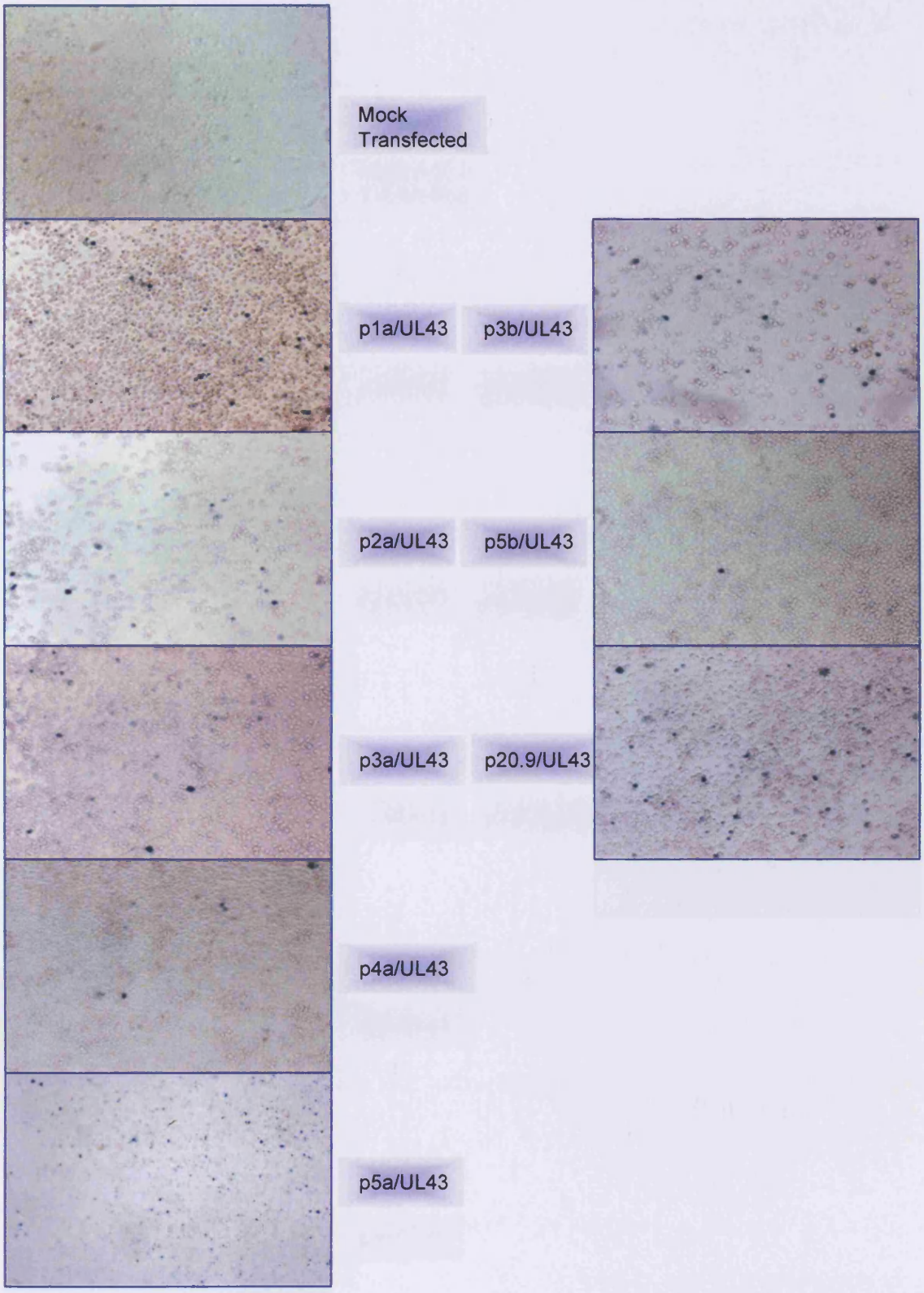


Figure 4-6c Photographs of transient transfections in ND7 cells – series 2 plasmids

5μg of each plasmid was transfected into ND7 cells, left for 48 hours and then stained with X-Gal to visualise *lacZ* expression.

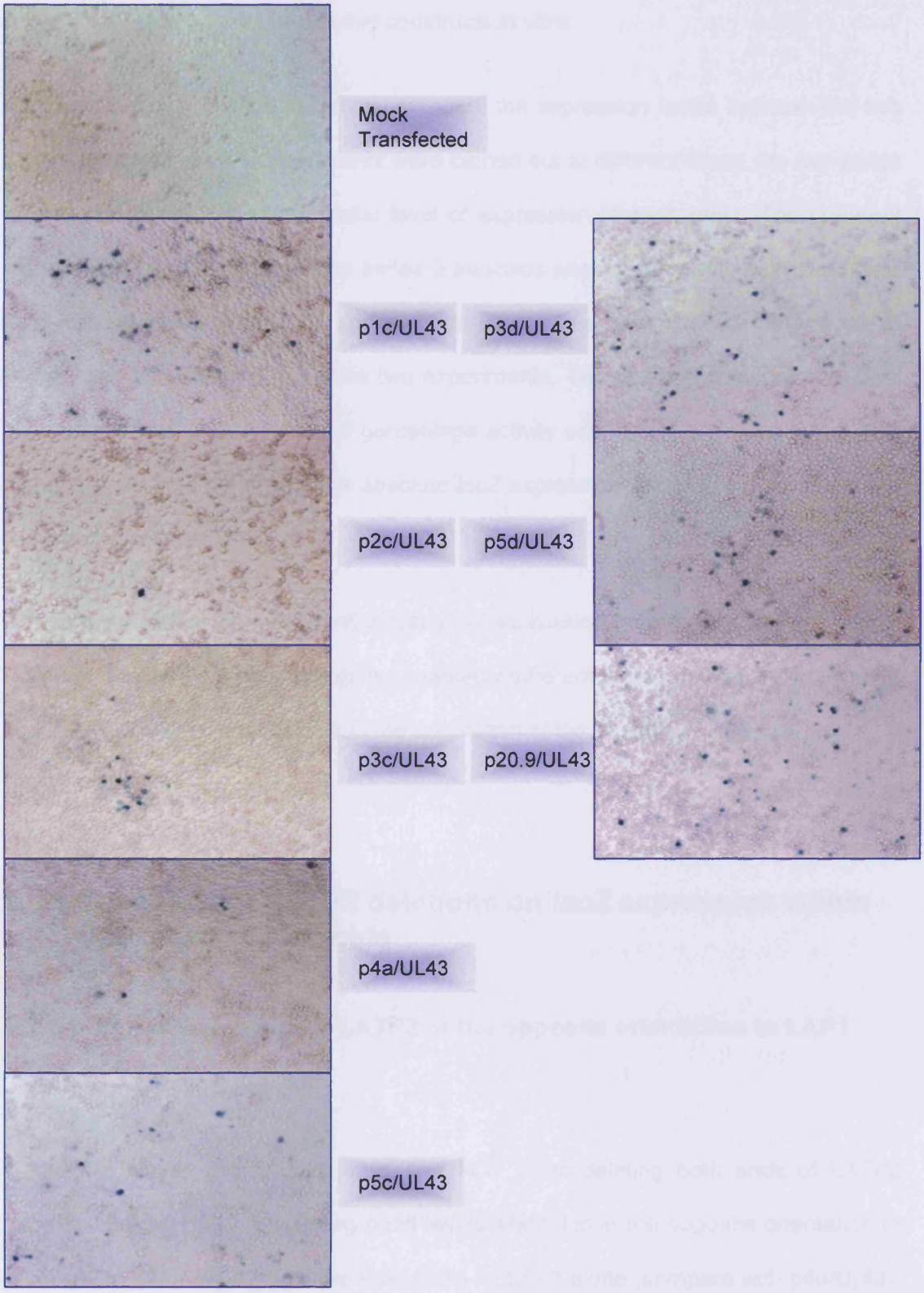


Figure 4-6d Photographs of transient transfections in ND7 cells – series 3 plasmids

5μg of each plasmid was transfected into ND7 cells, left for 48 hours and then stained with X-Gal to visualise *lacZ* expression.

The results of the *lacZ* quantification show the effect of deletions of the LATP2 region on *lacZ* expression from the plasmid constructs *in vitro.*

Although it is not possible to directly compare the expression levels between the two sets of plasmids, as the experiments were carried out at different times, the two series of plasmids seem to have a similar level of expression to each other. The apparent higher expression levels from the series 3 plasmids seen in the photographs in figure 4-6b compared to that from series 2 in figure 4-6a are due to differences in transfection efficiency between the two experiments. The fact that the control in both experiments is similar in terms of percentage activity confirms this (Figure 4-5a). This demonstrates the reason why the absolute *lacZ* expression values are not plotted, but displayed in relative terms.

The series 2 plasmids give a similar pattern of expression in both BHK and ND7 cells. However, the series 3 plasmids give a markedly different pattern of expression in BHK and ND7 cells. This must be due to the orientation of the region.

4.3.1 The effect of LATP2 deletions on *lacZ* expression within the series of plasmids

4.3.1.1 Series 2 plasmids – LATP2 in the opposite orientation to LAP1

(Paler green bars in figure 4-5)

Figure 4-5 shows that in both BHK and ND7 cells, deleting both ends of LATP2 reduces *lacZ* expression to background levels when it is in the opposite orientation to LAP1 (p2a/U_L43), even below the level given by LAP1 alone (compare with p4a/U_L43). Removing the ICP4 responsive region from 5'LATP2 abrogates enhancer activity (p3b/U_L43) and actually represses in ND7 cells. This is not seen however when it is removed from the full LATP2. All other deletions do not significantly alter *lacZ*

expression as compared to that when the full LATP2 is present. The control plasmid pR20.9 gives significantly higher ($p<0.001$ in BHKs, $p<0.05 - 0.001$ in ND7s) *lacZ* expression than any of the others, showing the effect of the MMLV LTR in the cassette.

4.3.1.2 Series 3 plasmids – LATP2 in the same orientation to LAP1

(Darker green bars in figure 4-5)

Deleting the ICP4 responsive region from the 5' half of LATP2 gives a significant ($p<0.001$) increase in *lacZ* expression compared to all other plasmids (except pR20.9/U_L43) when in the same orientation as LAP1 in BHK cells, but not ND7 cells. This increase in expression is not seen when the same region is deleted from the full LATP2 in BHK cells, but is in ND7 cells. As ICP4 is not present in the transfection experiments, this effect must be due to deletion of a differently repressive element at this location. The mid section of LATP2 also allows increased *lacZ* expression when in ND7 cells compared to that from the whole LATP2 ($p<0.001$). This would suggest that a repressor, active in neuronal cells, has been removed from this section of LATP2. However, this same reprieve from repression might be expected in either the plasmid with the 5' half of LATP2 or the 3' half. The fact that although a certain increase in expression is seen with both of these plasmids compared to the full LATP2-containing plasmid, but not to the same extent suggests that there are possibly repressive elements in each end of LATP2.

4.3.2 The effect of orientation of LATP2 deletions on *lacZ* expression

In the transfections carried out in BHK cells (figure 4-5a), differences between the LATP2 fragments in different orientations are only seen in the mid section of LATP2 and the 5' LATP2ΔICP4 fragment. Both of these LATP2 fragments allow barely any transgene expression from LAP1 when placed in the opposite orientation to LAP1, but when in the same orientation are capable of enhancing expression at least as well as the full LATP2 region. Therefore these two fragments of LATP2 must be missing an element essential for enhancer activity when in the opposite orientation to LAP1. The effect of ICP4 in these experiments is obviously irrelevant as there is none present, therefore this stretch of DNA in the 5' LATP2 fragment must also code for a recognition element essential for LATP2 activity. The fact that when this is deleted from the full LATP2, expression from LAP1 is still allowed suggests that this element must work in concert with another not present in the 5' half and perhaps not in the mid-section. It is a little strange that there is directionality in these elements, as classic enhancers are orientation independent. LATP2 may contain a complicated enhancer that requires a number of elements to work optimally.

The same effect of orientation is also seen with these two plasmids in ND7 cells, to a greater extent with the mid fragment of LATP2 and to a lesser extent with the 5' LATP2ΔICP4 fragment, again pointing to neuronal-responsive elements being present in these LATP2 fragments. The biggest difference in orientation effect between BHK and ND7 cells is that of the full LATP2ΔICP4. This region in either orientation allows similar expression levels of *lacZ* to the full LATP2 in BHK cells, however in ND7 cells, placing the full LATP2ΔICP4 in the same orientation as LAP1 gives a significant ($p<0.001$) increase in *lacZ* expression over that from the full LATP2. If there is a repressor of LATP2 activity within the stretch of DNA deleted then it is neuronal-specific.

A summary of the results from the *in vitro* transfection experiments is given as follows:

- All LATP2 fragments contain some enhancer activity, but this is in some cases directional.
- Directionality is most noticeable for the mid LATP2, 5' LATP2ΔICP4 and full LATP2ΔICP4 fragments.
- Mid LATP2 and 5'LATP2ΔICP4 are repressive in the opposite of their natural orientation with respect to LAP1, but enhance considerably in the natural orientation.
- Deleting the ICP4 responsive region considerably boosts expression in the correct orientation; however, these effects are variable between BHK and ND7 cells.
- It appears that central regions of LATP2 have most enhancer functions, but that these may be counterbalanced by repressive elements at either end.

The fact that the activity of some fragments of LATP2 appears to differ whether in fibroblasts or neuronal-derived cells is perhaps not surprising as the natural host for HSV1 is neurons. As stated before however, even *in vitro* neuronal systems do not contain all of the factors present *in vivo*. Therefore the effect of the LATP2 deletions was hoped to become clearer when in the context of the animal model.

4.4 VECTOR CONSTRUCTION

Viral vectors were again produced using the 1764 virus backbone, as described in section 3.4, and cassettes inserted into the U_L43 gene.

The thirteen LATP2 deletion plasmids were linearised with *Xmn1* and co-transfected with 1764/U_L43/CMV/GFP viral DNA (Figure 4-7). Recombinant vectors containing the cassettes were identified by loss of GFP and gain of *lacZ* marker genes and plaque purified. Insertion of the cassettes was confirmed by Southern blot analysis (see Figure 4-8a-b).

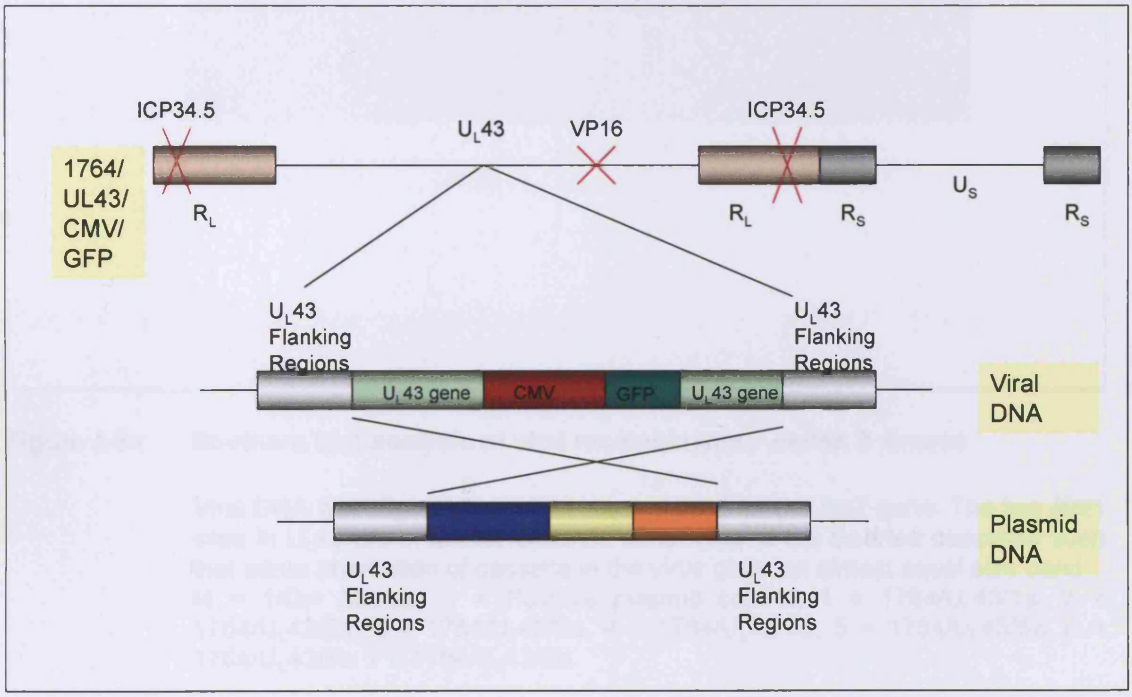


Figure 4-7 Homologous recombination to produce recombinant HSV vectors

Plasmid DNA was co-transfected with 1764/U_L43/CMV/GFP viral DNA into BHK cells. During lytic replication, homologous recombination occurs between the U_L43 flanking regions present in both plasmid and viral DNA.

Figure 4-8a **Southern blot analysis of viral recombinants – series 2 viruses**

Viral DNA was digested with *Not*1 and probed for the *lacZ* gene. The two *Not*1 sites in U_L43 are of similar distance either side of the inserted cassettes such that either orientation of cassette in the virus gives an almost equal size band. M = 1Kb+ Marker. + = Positive plasmid control, 1 = 1764/U_L43/1a, 2 = 1764/U_L43/2a, 3 = 1764/U_L43/3a, 4 = 1764/U_L43/4a, 5 = 1764/U_L43/5a, 6 = 1764/U_L43/3b, 7 = 1764/U_L43/5b.

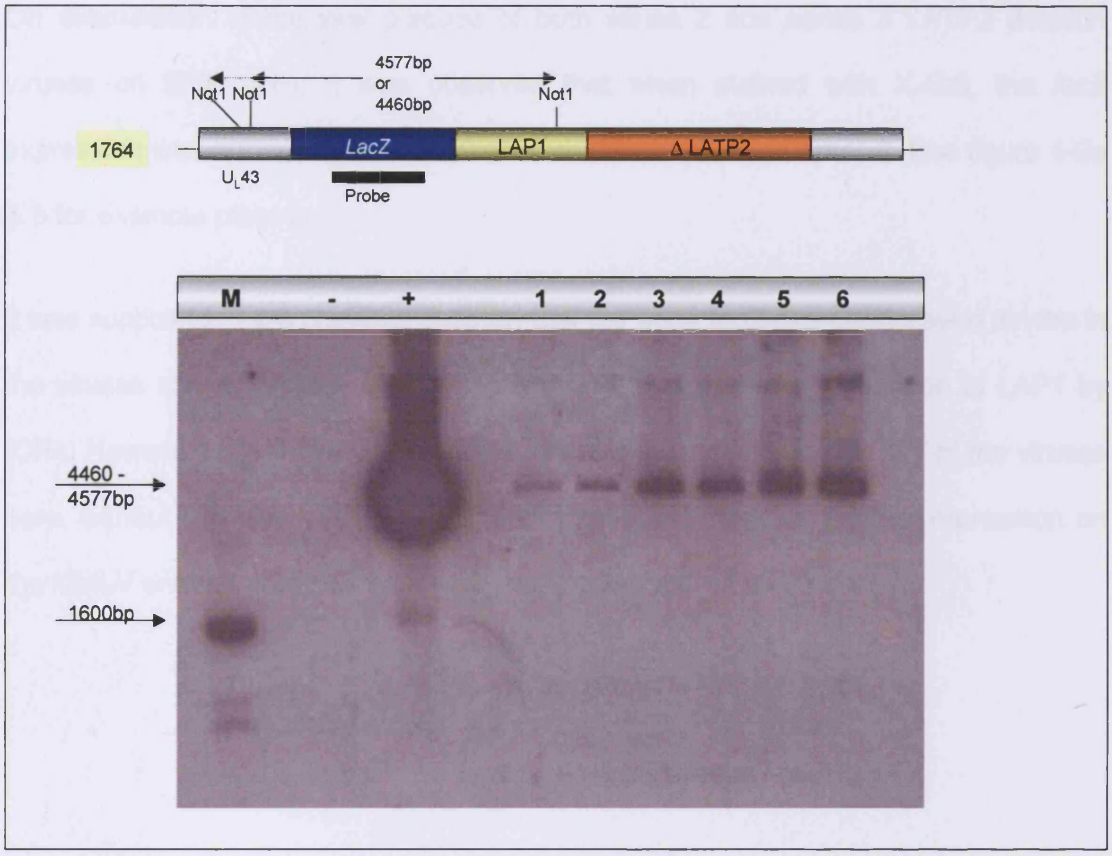


Figure 4-8b Southern blot analysis of viral recombinants – series 3 viruses

Viral DNA was digested with *Not*1 and probed for the *lacZ* gene. The two *Not*1 sites in U_L43 are of similar distance either side of the inserted cassettes such that either orientation of cassette in the virus gives an almost equal size band. M = 1Kb+ Marker, + = Positive plasmid control, 1 = 1764/U_L43/1c, 2 = 1764/U_L43/2c, 3 = 1764/U_L43/3c, 4 = 1764/U_L43/5c, 5 = 1764/U_L43/3d, 6 = 1764/U_L43/5d.

4.5 *IN VITRO* TESTING OF RECOMBINANT VECTORS

4.5.1 Viral Plaque Characteristics

On examination of the viral plaques of both series 2 and series 3 LATP2 deletion viruses on BHK cells, it was observed that when stained with X-Gal, the *lacZ* expression was stronger than that seen from the viruses in chapter 3. See figure 4-9a & b for example plaques.

It was supposed in the previous chapter, that the weak *lacZ* expression seen *in vitro* in the viruses containing the MMLV LTR and GFP was due to a repression of LAP1 by ICP4. However, as the weak expression is not seen in the viral plaques of the viruses here, without the MMLV LTR and GFP, it might have been a result of repression on the MMLV enhancer activity by a viral protein, *in vitro*.

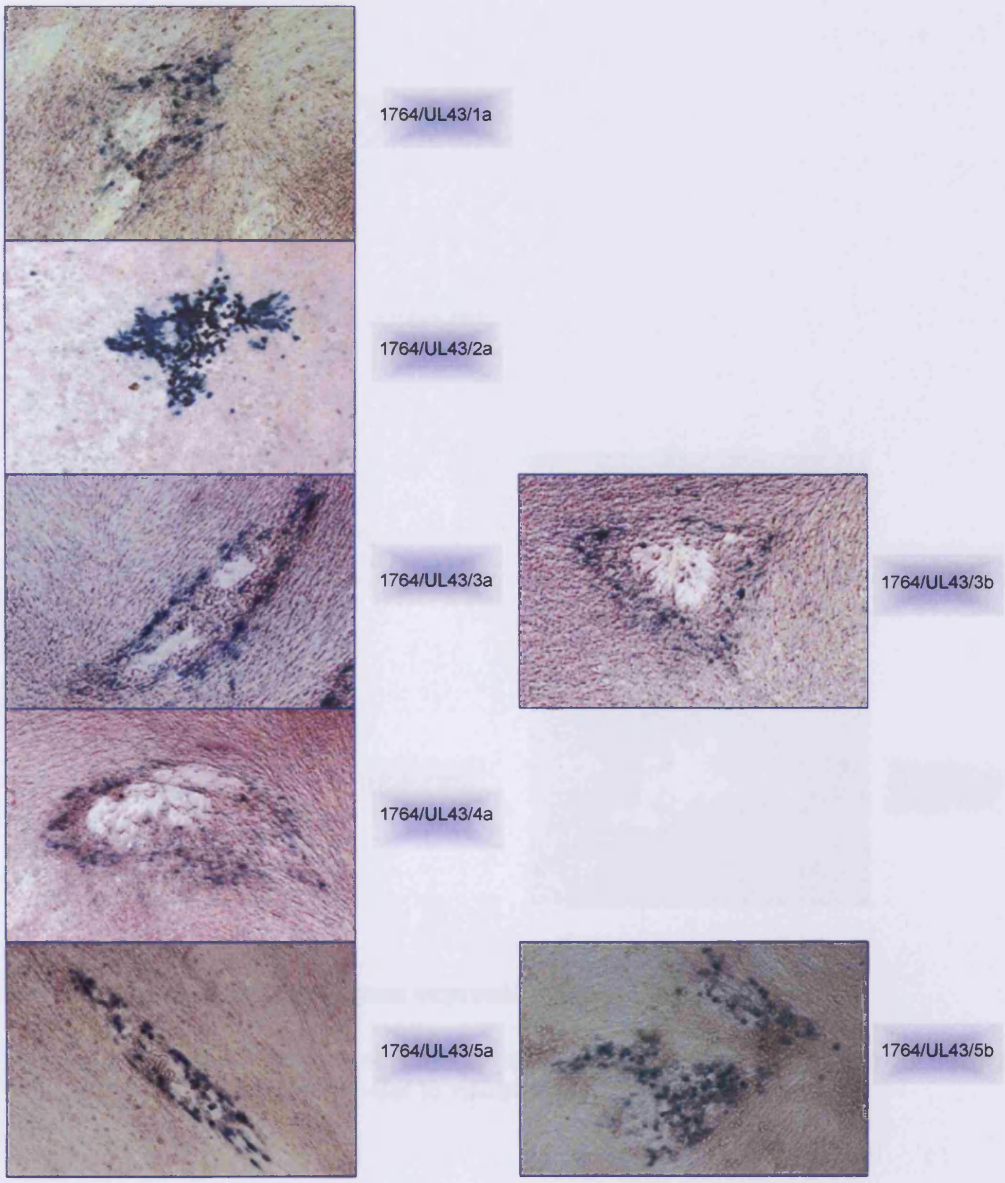


Figure 4-9a Example plaques expressing *lacZ*– series 2 viruses

BHK cells were infected with virus and plaques allowed to develop before staining with X-Gal to visualise *lacZ* expression.

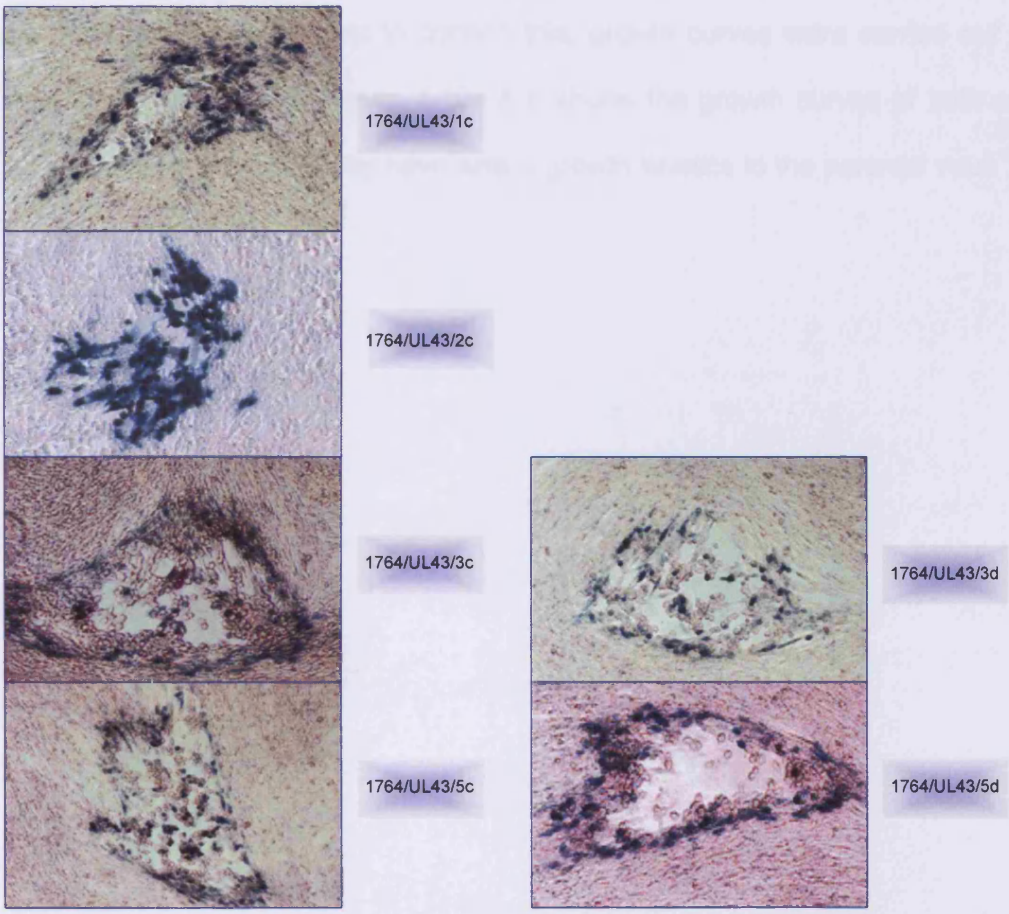


Figure 4-9b Example plaques expressing *lacZ*– series 3 viruses

BHK cells were infected with virus and plaques allowed to develop before staining with X-Gal to visualise *lacZ* expression.

4.5.2 Growth curves

It would be expected that the growth characteristics of the two series of LATP2 deletion viruses would be the same as that of the parental 1764 virus, as those in the previous chapter were. Just to confirm this, growth curves were carried out in BHK cells, at an MOI of 0.1. Figure 4-10a & b shows the growth curves of both series of viruses and as expected they have similar growth kinetics to the parental virus 1764.

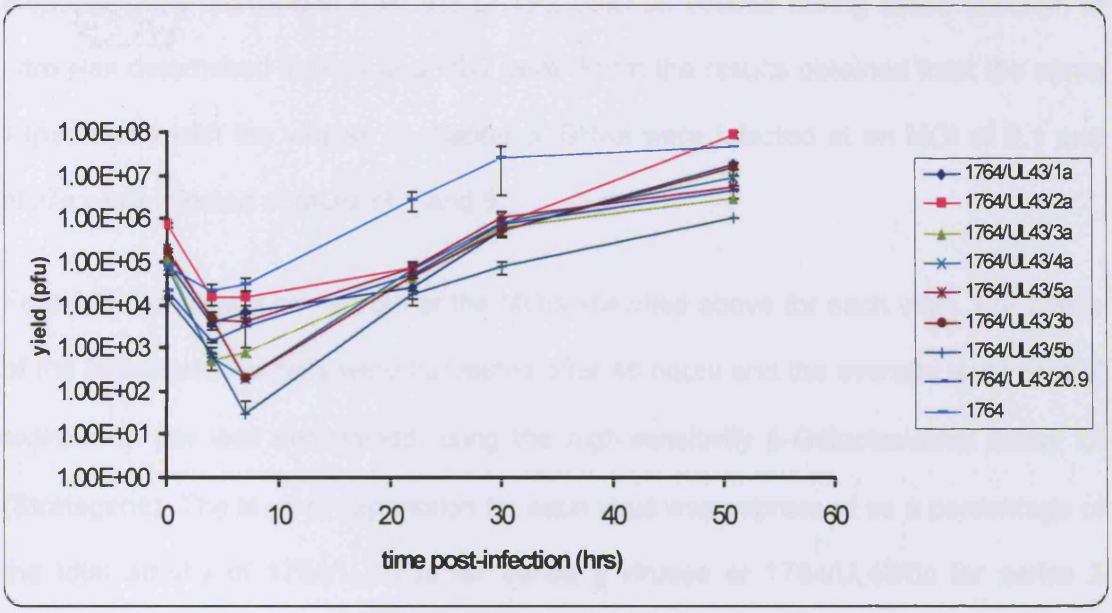


Figure 4-10a Growth curves of series 2 viruses

BHKs were infected at an MOI of 0.1 and harvested at 4, 7.5, 22, 31 and 51 hours and titred in a standard viral plaque assay.

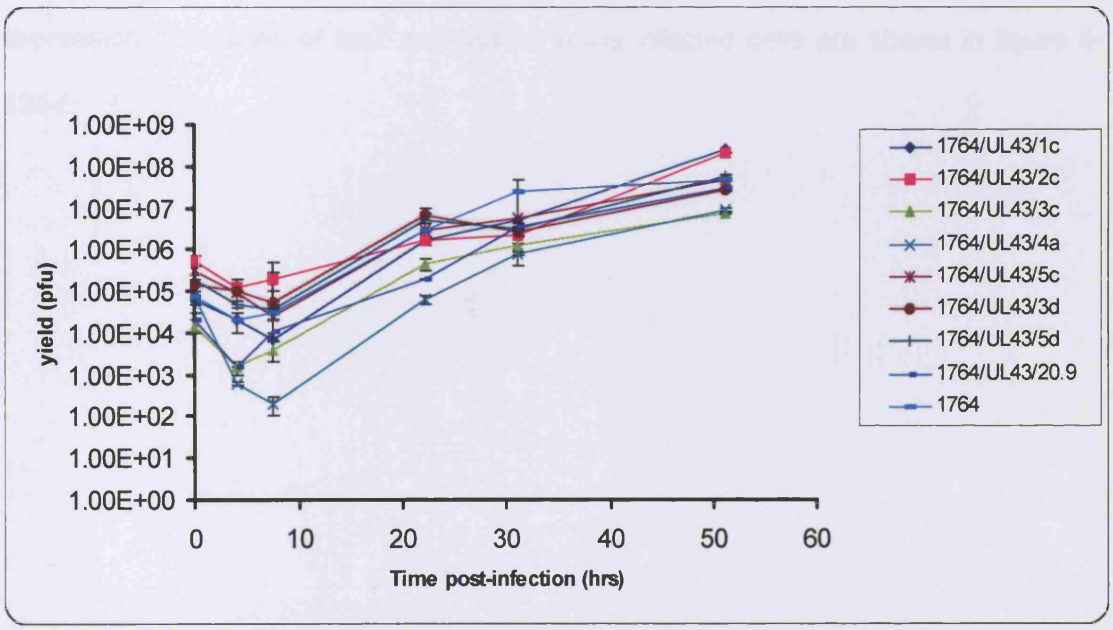


Figure 4-10b Growth curves of series 3 viruses

BHKs were infected at an MOI of 0.1 and harvested at 4, 7.5, 22, 31 and 52 hours and titred in a standard viral plaque assay.

4.5.3 Infection of Cell Lines

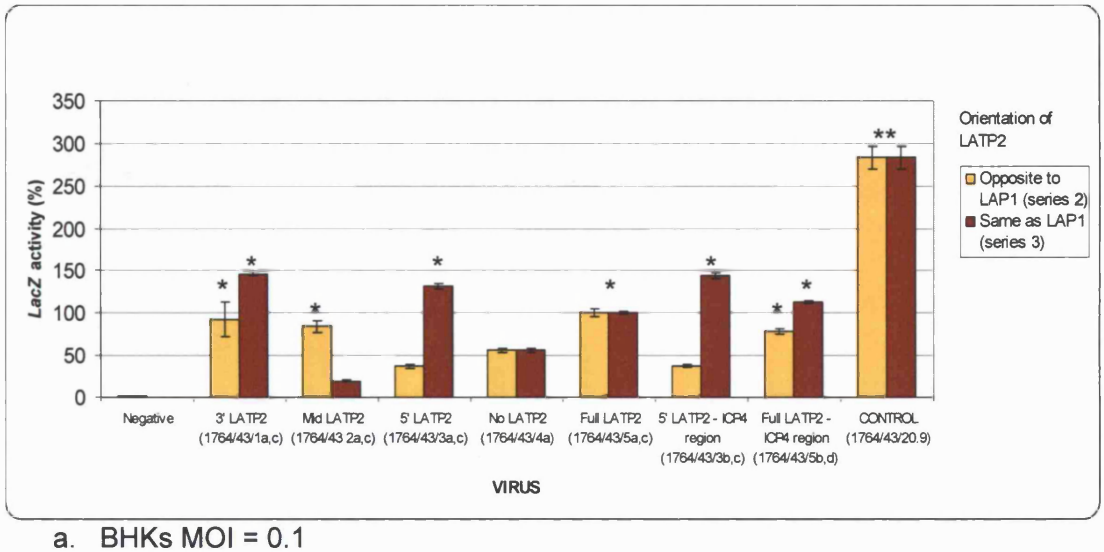
Reporter gene expression from the LATP2 deletion viruses during acute infection *in vitro* was determined in BHK and ND7 cells. From the results obtained from the same experiments with the viruses in chapter 3, BHKs were infected at an MOI of 0.1 and ND7s were infected at MOIs of 1 and 5.

Four infections were carried out at the MOIs specified above for each virus. For 3 sets of the infections, the cells were harvested after 48 hours and the average level of *lacZ* expression per well determined using the high-sensitivity β-Galactosidase assay kit (Stratagene). The level of expression for each virus was expressed as a percentage of the total activity of 1764/U_L43/5a for series 2 viruses or 1764/U_L43/5c for series 3 viruses. As the two virus series were infected at the same time, the data for 1764/U_L43/4a (No LATP2) and 1764/U_L43/20.9 (control) is the same for each series. The results are shown in figure 4-11a-c.

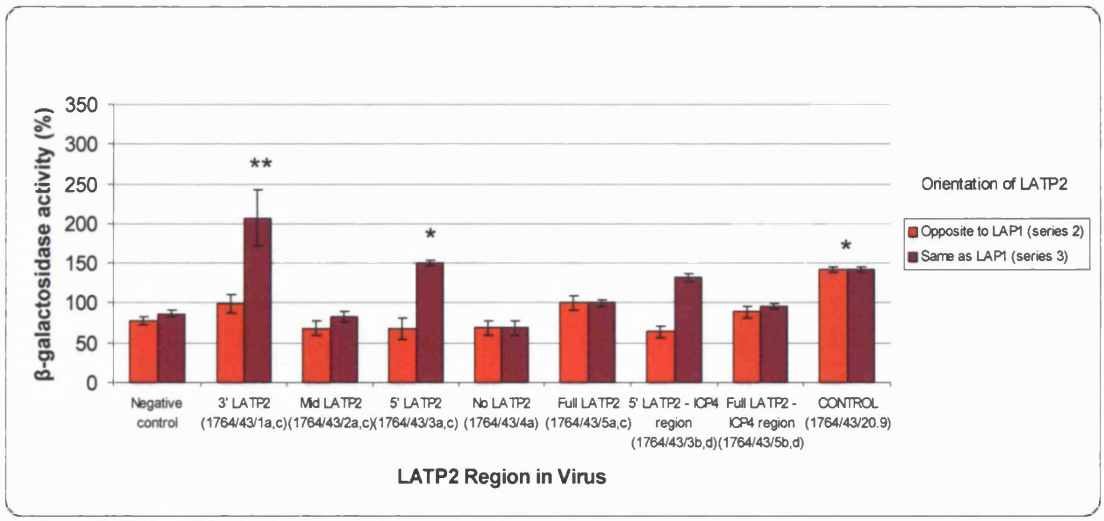
The fourth set of infections was stained with X-Gal after 48 hours, to visualise *lacZ* expression. Examples of *lacZ* expression in the infected cells are shown in figure 4-12a-f.

Figure 4-11 ***In vitro*** **β-Galactosidase assays.**

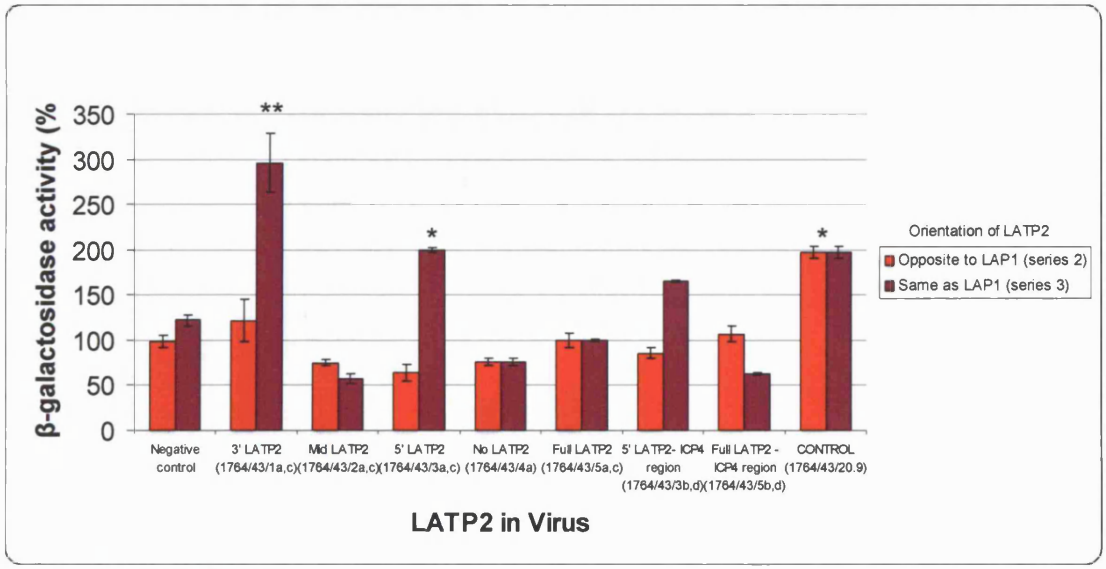
BHKs (a) and ND7s (b, c) were infected with virus at different MOIs and left for 48 hours before assay. The positive control is the 1764/U_L43/20.9 virus. The negative control is a 1764 virus expressing GFP in the U_L43 locus.
(* - $p<0.05$, ** - $p<0.01$)



a. BHKs MOI = 0.1



b. ND7s MOI = 1



c. ND7s MOI = 5

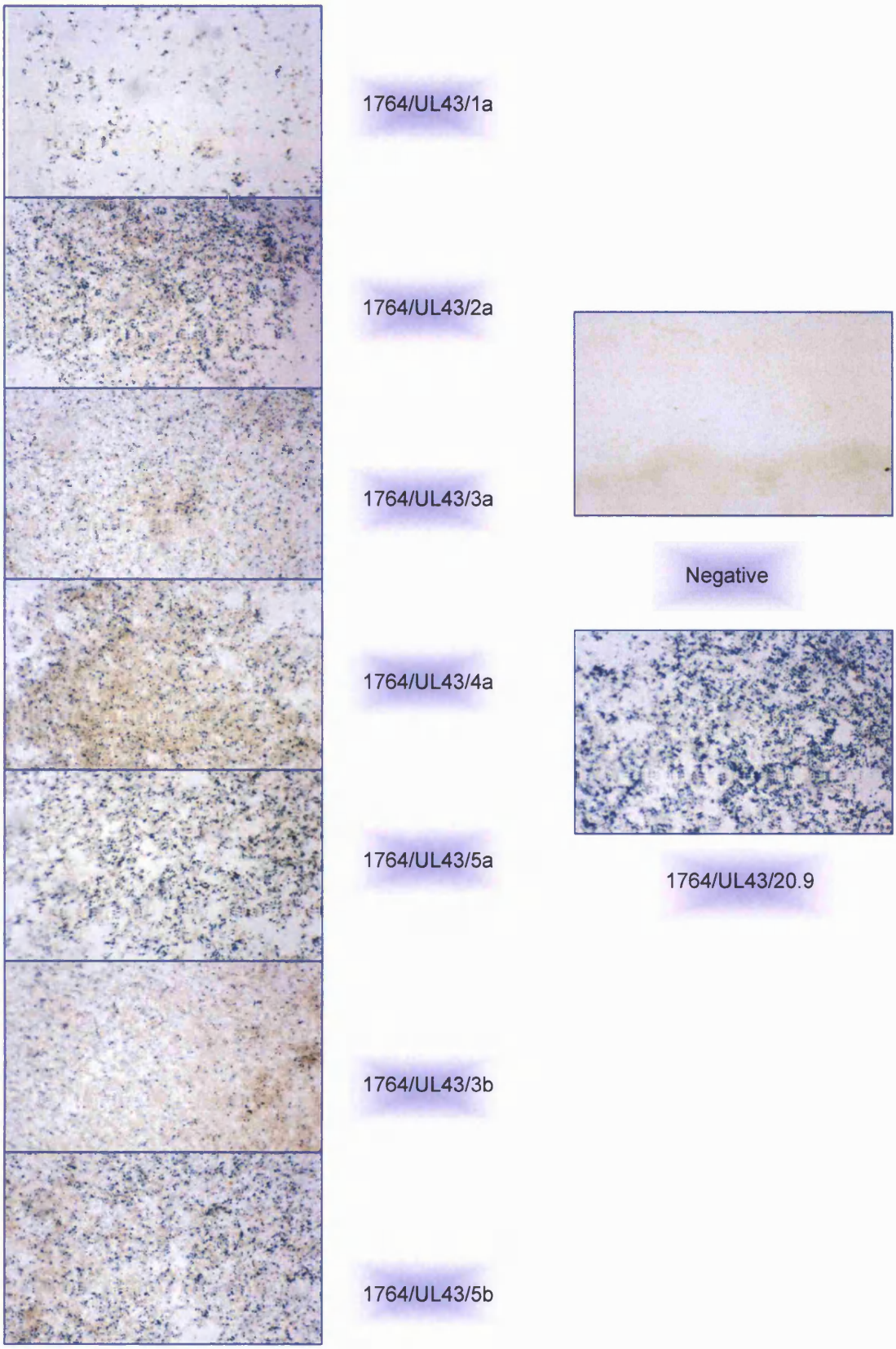


Figure 4-12a Examples of infected BHK cells – series 2 viruses

BHK cells were infected at an MOI of 0.1 and stained for *lacZ* 48 hrs p.i.

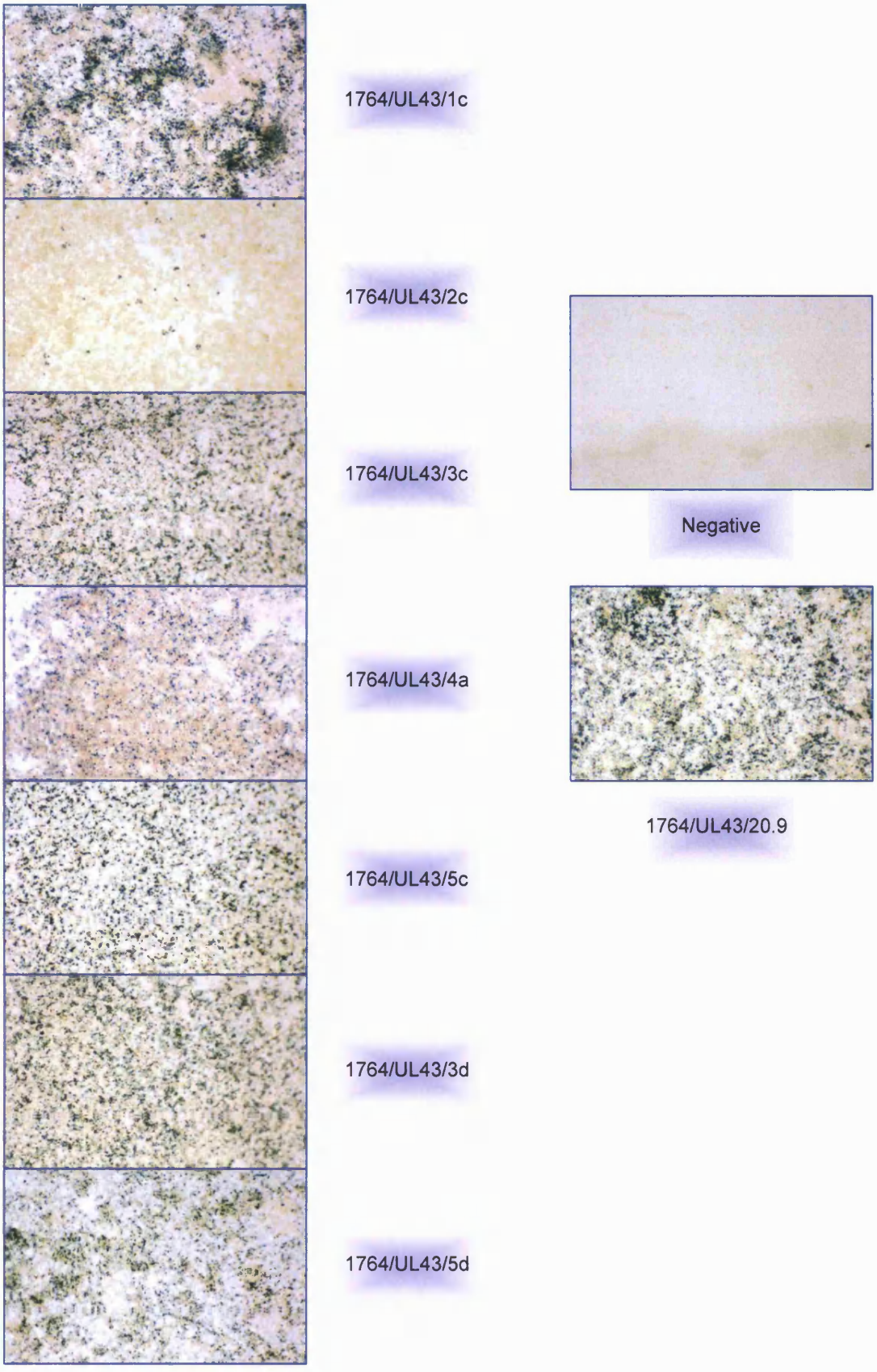


Figure 4-12b Examples of infected BHK cells – series 3 viruses
BHK cells were infected at an MOI of 0.1 and stained for *lacZ* 48 hrs p.i.

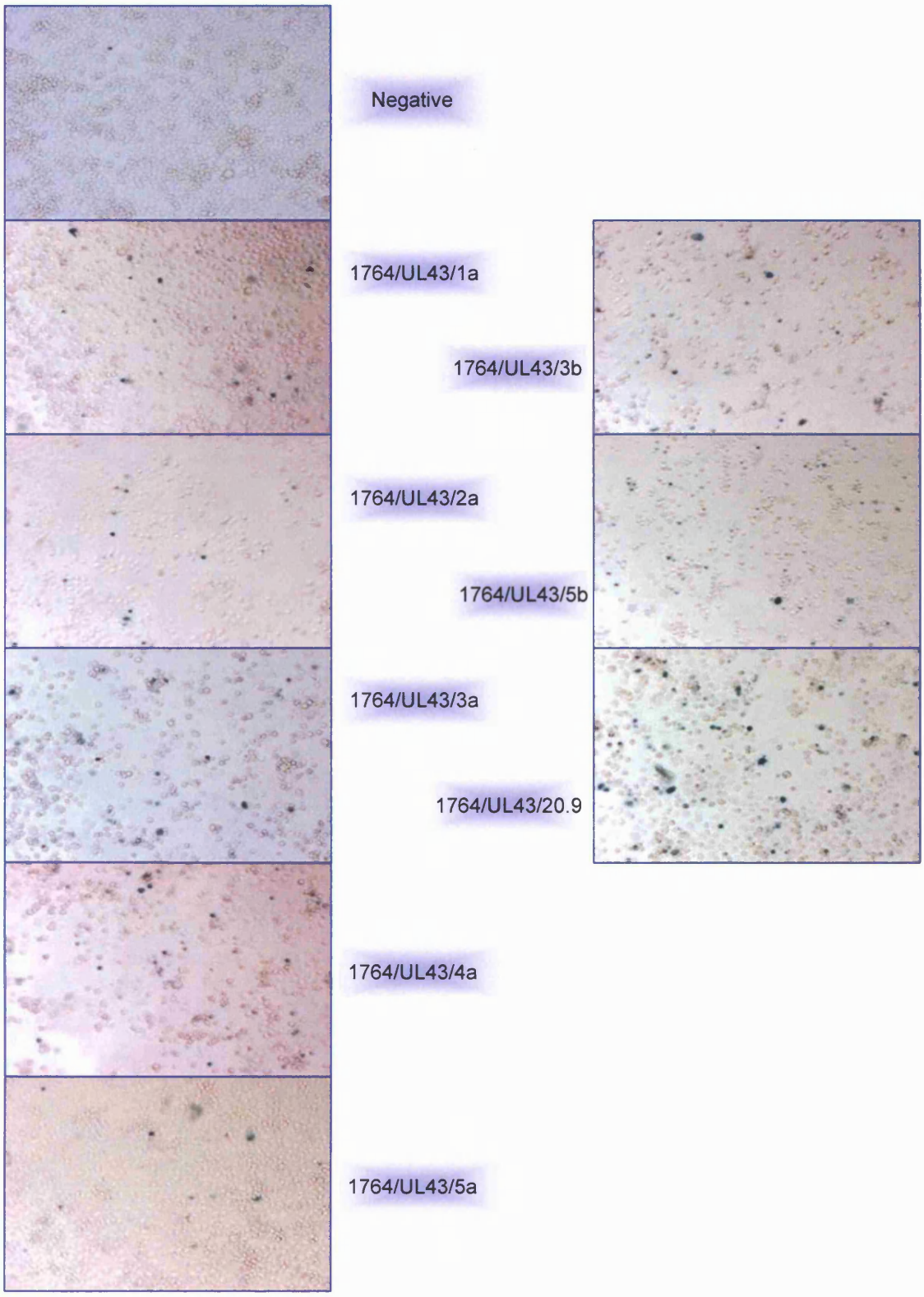


Figure 4-12c Examples of infected ND7cells (MOI = 1) – series 2 viruses

ND7 cells were infected at an MOI of 1 and stained for *lacZ* 48 hrs p.i.

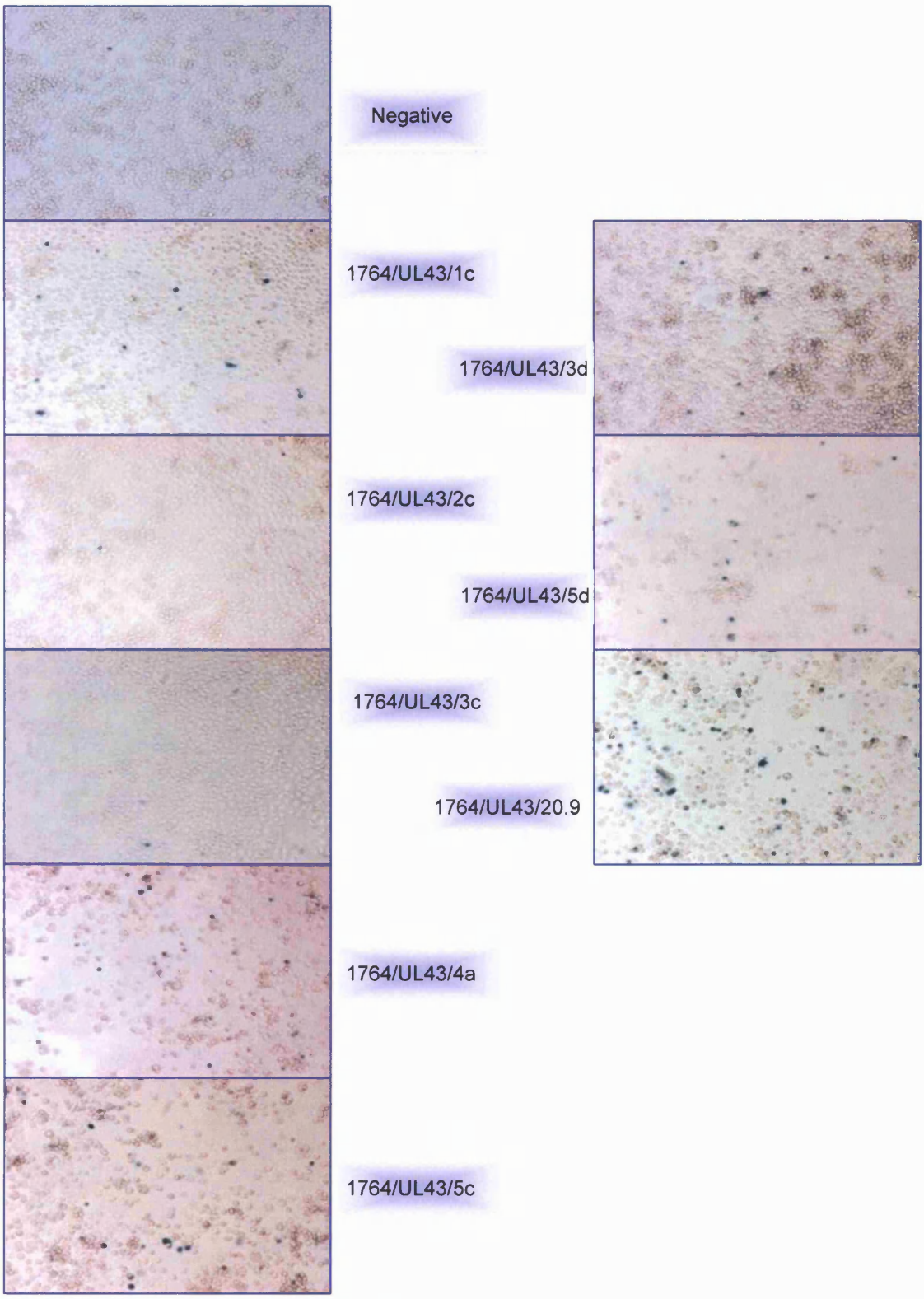


Figure 4-12d Examples of infected ND7cells (MOI = 1) – series 3 viruses

ND7 cells were infected at an MOI of 1 and stained for *lacZ* 48 hrs p.i.

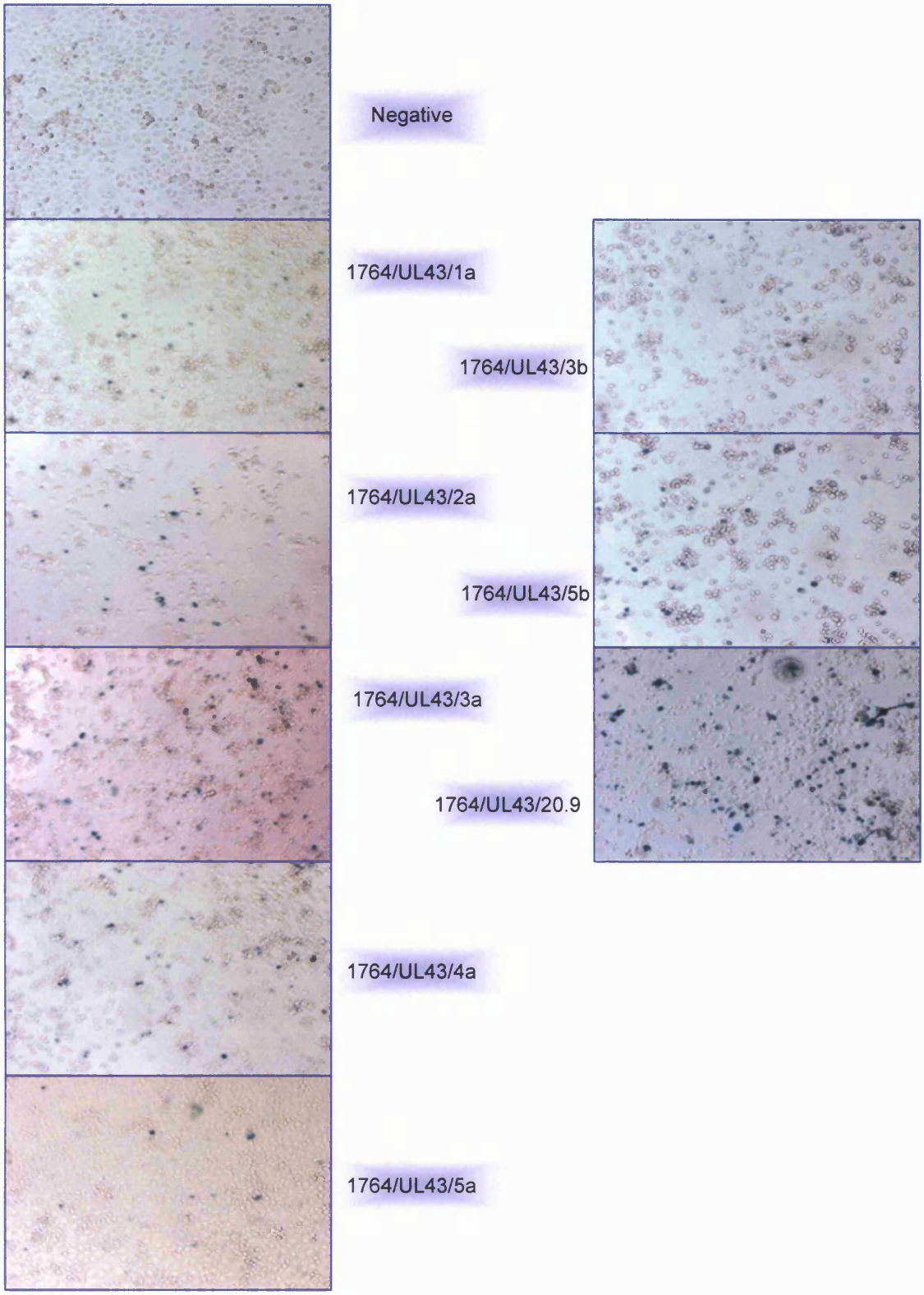


Figure 4-12e Examples of infected ND7cells (MOI = 5) – series 2 viruses

ND7 cells were infected at an MOI of 5 and stained for *lacZ* 48 hrs p.i.

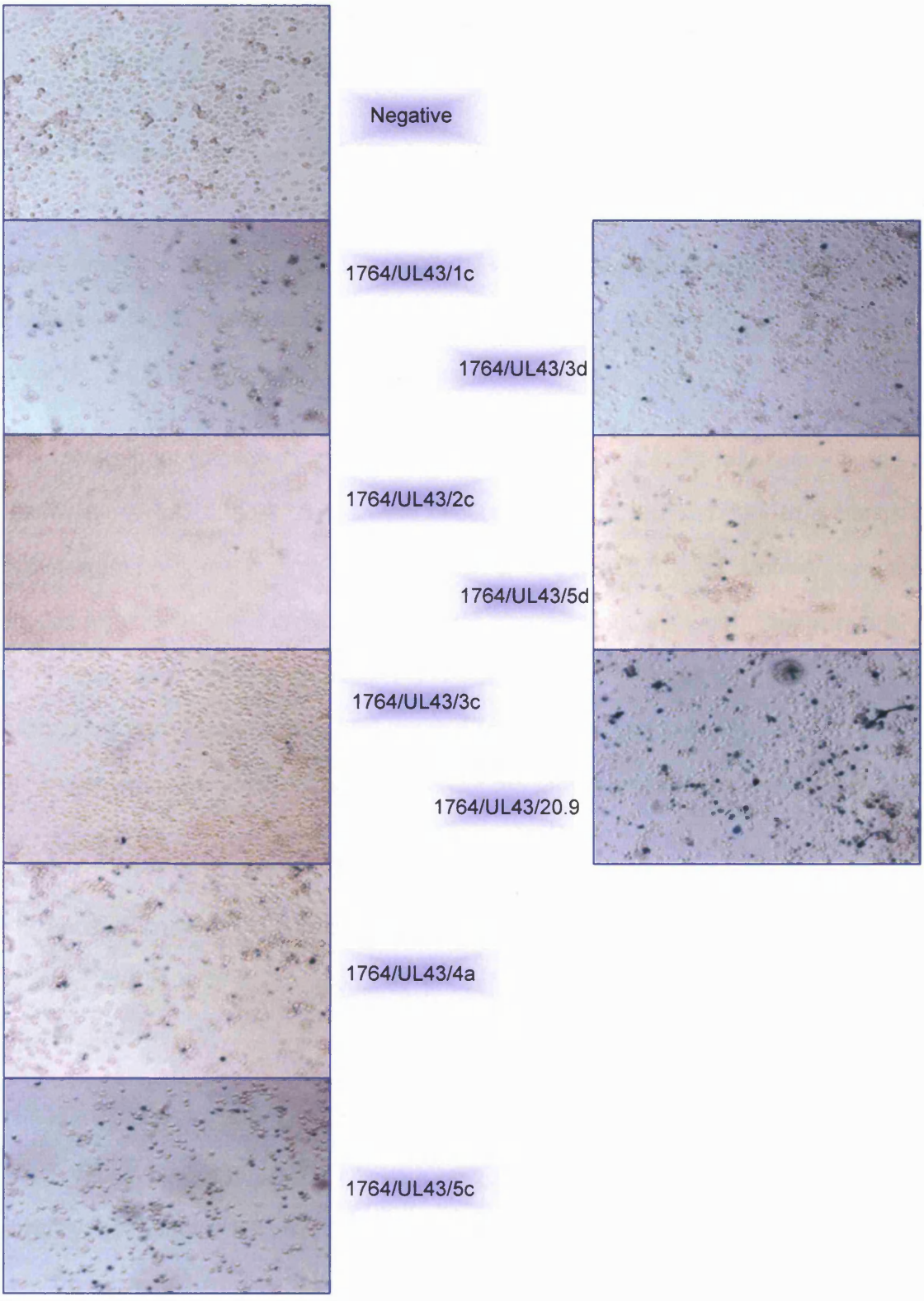


Figure 4-12f Examples of infected ND7cells (MOI = 5) – series 3 viruses

ND7 cells were infected at an MOI of 5 and stained for *lacZ* 48 hrs p.i.

4.5.4 The effect of LATP2 deletions on *lacZ* expression within the series of viruses

4.5.4.1 Series 2 viruses – LATP2 in the opposite orientation to LAP1

The series 2 viruses only produced enough *lacZ* to be detectable by the *lacZ* assay when infected in BHK cells. The ND7 infections, although showing some *lacZ* expression in the photographs in figure 4-12c-f, did not produce enough *lacZ* from most viruses to be detectable in the *lacZ* assay.

The results from figure 4-11 in BHK cells show that when LATP2 is in the opposite orientation to LAP1 the 3' half or mid section of LATP2 are sufficient to enhance activity from LAP1 at the same level as the full LATP2 ($p<0.05$). *LacZ* expression from viruses with the 5' half of LATP2 is no greater than that from just LAP1. Removing the ICP4 responsive region from either the 5' half or full LATP2 has no effect on *lacZ* expression from these viruses. The control virus, containing the MMLV LTR again gives greater *lacZ* expression than that without this promoter/enhancer ($p<0.01$).

4.5.4.2 Series 3 viruses – LATP2 in the same orientation as LAP1

The series 3 viruses *in vitro* produced slightly more *lacZ* on the whole than the series 2 viruses. The BHK infections produced enough *lacZ* to be detected, but only a few viruses gave enough *lacZ* in the ND7 infections to be detectable in the *lacZ* assay.

The results in figure 4-11 show that when LATP2 is in the same orientation as LAP1 the 3' and 5' halves of LATP2 are both able to enhance *lacZ* expression to a level seen by that from the full LATP2 in BHK cells (all $p<0.05$ compared to LAP1 only) and to a higher level than that from the full LATP2 in ND7 cells at an MOI of 1. At the higher MOI of 5 in ND7 cells, the *lacZ* expression level from these two viruses increased further with respect to the full LATP2. The mid section of LATP2 is not able to enhance *lacZ* expression *in vitro* above the level of LAP1 alone when in the same

orientation as LAP1. Again removing the ICP4 responsive region from either the 5' half or full LATP2 has no effect on *lacZ* expression from these viruses.

4.5.5 The effect of orientation of LATP2 deletions on *LacZ* expression

The 5' half of LATP2 allows the same or higher level of transgene expression as the full LATP2 only when in the same orientation as LAP1. The 3' half allows the same level of expression when in either orientation in BHK cells, but can only enhance when in the same orientation as LAP1 in ND7 cells. The mid section of LATP2 can only enhance *lacZ* expression from LAP1 when in the opposite orientation to LAP1 in BHK cells, but not at all in ND7s. This could suggest an unusual uni-directional enhancer activity located in this section. Elements located in LATP2 that are deleted from the 1764/UL43/2c virus (mid section of LATP2) i.e. in either end of the region appear to be required for activity when LATP2 is in the same orientation as LAP1.

A similar study carried out whilst this work was in progress (Berthomme *et al.* 2001) on viruses with similar deletions to those described here gave a different result *in vitro*. All of the deletion fragments were in the same orientation as LAP1, so most comparable with the series 3 viruses described here. Neither the 3' half or the 5' half of the region were capable of enhancing as high an expression level of *lacZ* as the full region in either BHK or ND7 cells so they predicted that elements throughout the region are required for full enhancer activity. The difference in the viruses used in that study and here is in the position of LATP2 with relation to LAP1. Berthomme *et al.* used deletions of the LATP2 region in its native locus, such that LATP2 was downstream of LAP1, whereas here, LATP2 is upstream of LAP1. This presumably accounts for the differing expression profiles of the viruses *in vitro*. This group also carried out their infections of ND7 cells at an MOI of 10, which in hindsight perhaps would have been wise here as the level of *lacZ* expression was mostly too low in ND7 cells at an MOI of 5 to obtain any useful data.

4.6 *IN VIVO* TESTING OF RECOMBINANT VECTORS

To investigate the activity of LATP2 in the viruses described further, *in vivo* experiments were carried out.

4.6.1 Testing in the PNS

3-week-old Balb/C or Balb/C SCID mice were injected into the left footpad with 20μL of each virus at a titre of 1 x 10^8 pfu/mL (Total virus injected = 5 x 10^6 pfu). The dorsal root ganglia from the lumbar region ipselateral to the injection site were removed after 3 days (Balb/C) and 1 month (Balb/C SCID). DRG were post-fixed and stained with X-Gal. *LacZ*-positive neurons from DRG at positions L3-L6 were then counted and scored depending on the intensity of blue stain due to the two populations of stained neurons as explained in section 3.6.1.

4.6.1.1 LATP2 activity 3 days post-injection

Results for *lacZ* expression at 3 days post-injection (p.i.) are presented in figure 4-13 and table 4-2. Selected ganglia from each virus series are shown in figure 4-14a & b.

VIRUS	PALE NEURONS	DARK NEURONS	WEIGHTED TOTAL SCORE	S.E.M.
1764/UL43/1a (3' Half LATP2)	18.6	2.5	23.6	2.67
1764/UL43/2a (Mid LATP2)	39.9	7.6	55.1	5.83
1764/UL43/3a (5' Half LATP2)	20.1	3	26.1	3.06
1764/UL43/4a[1] (No LATP2)	18.2	2.1	22.4	3.06
1764/UL43/5a (Full LATP2)	27.8	3.5	34.8	5.43
1764/UL43/3b (5' LATP2ΔICP4)	8	1	10	1.93
1764/UL43/5b (Full LATP2ΔICP4)	24.3	3.2	30.7	5.94
1764/UL43/20.9[1] (Control)	41.9	52.1	146	17.4

VIRUS	PALE NEURONS	DARK NEURONS	WEIGHTED TOTAL SCORE	S.E.M.
1764/UL43/1c (3' Half LATP2)	28.4	7.1	42.6	5.14
1764/UL43/2c (Mid LATP2)	1.3	0.1	1.5	0.6
1764/UL43/3c (5' Half LATP2)	20	2.7	25.4	2.52
1764/UL43/4a[1] (No LATP2)	18.2	2.1	22.4	3.06
1764/UL43/5c (Full LATP2)	32.6	7.3	47.2	7.6
1764/UL43/3d (5' LATP2ΔICP4)	35.2	5.9	47	3.69
1764/UL43/5d (Full LATP2ΔICP4)	40.1	7.6	55.3	5.6
1764/UL43/20.9[1] (Control)	41.9	52.1	146.1	17.43

Table 4-2 Averaged counts of *lacZ*-positive neurons 3 days p.i.

LacZ positive neurons from ganglia L3 – L6 were recorded per animal, and scored according to whether staining for *lacZ* was pale or dark (n=10). Weighted total score = (# pale neurons + 2 times # of dark neurons)

[1] Data for 1764/UL43/4a and 1764/UL43/20.9 is the same for both series of viruses

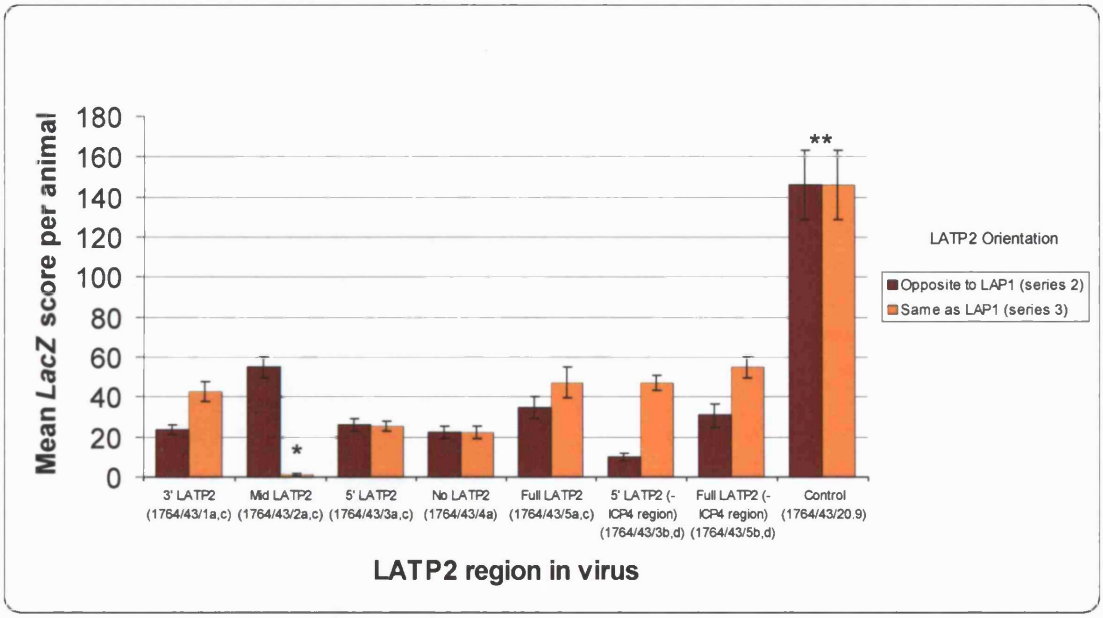


Figure 4-13 ***LacZ* expression from LATP2-deletion viruses 3 days post-injection in Balb/C mice**

Weighted *lacZ*-positive neuron scores give a semi-quantitative measure of *lacZ* expression from the LATP2 deletion viruses.
(** - $p<0.001$ * - $p<0.01$)

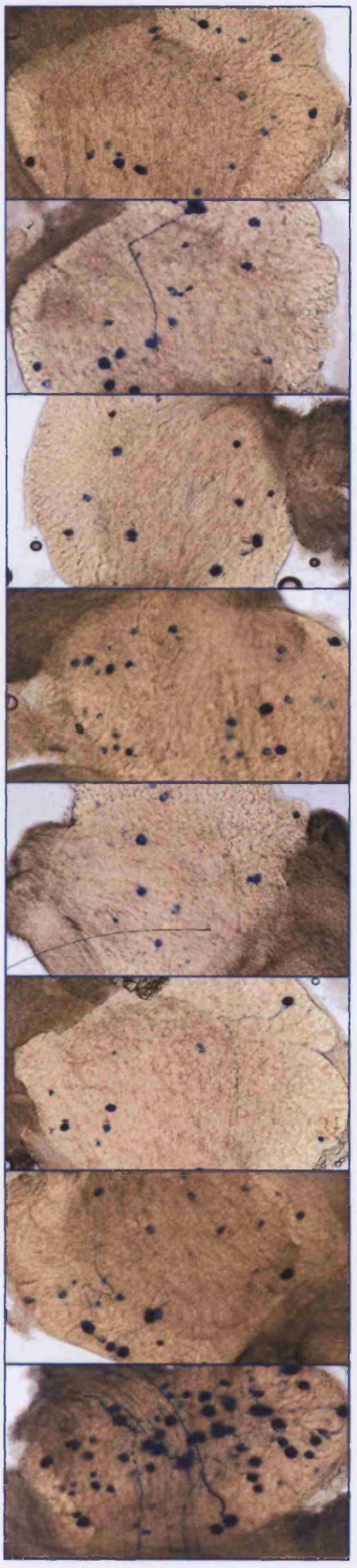


Figure 4-14a **Representative ganglia 3 days post-injection showing *lacZ* expression – series 2 viruses.**

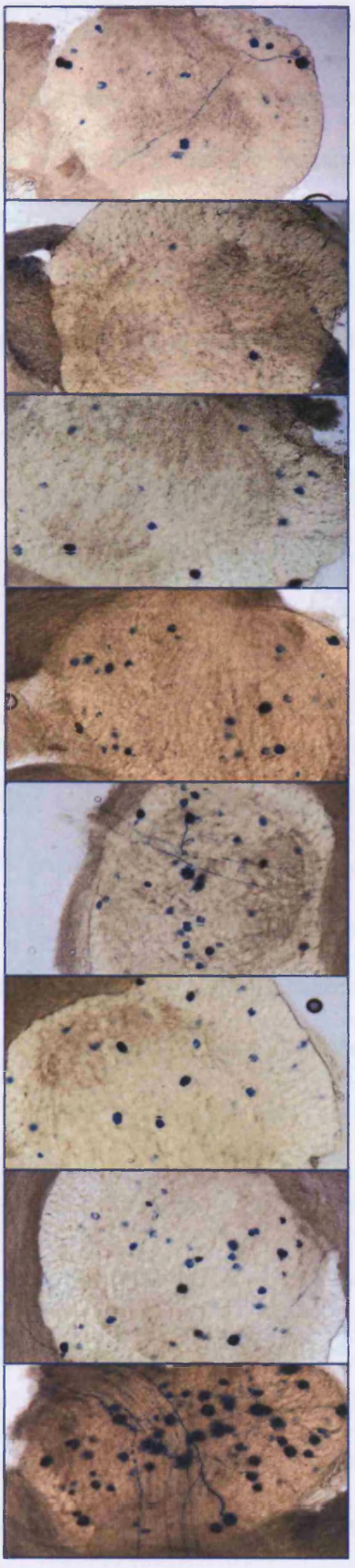


Figure 4-14b **Representative ganglia 3 days post-injection showing *lacZ* expression – series 3 viruses.**

4.6.1.1.1 Series 2 viruses – LATP2 in the opposite orientation to LAP1

Figure 4-13 illustrates the effect of deletions of the LATP2 region on *lacZ* expression *in vivo* at 3 days p.i. The first striking result is the higher level of *lacZ* expression given from the 1764/U_L43/20.9 virus, containing the MMLV LTR compared to the viruses without this promoter/enhancer. This shows that the MMLV LTR linked to LATP2 and LAP1 provides 4 times the expression level of that from just LAP1 and LATP2.

The virus containing the mid section of LATP2 (1764/U_L43/2a) appears to give a slightly higher expression level than the rest of the series, but this is only significantly more than 1764/U_L43/4a, the virus with no LATP2 ($p<0.05$) and 1764/UL43/3b, the virus with the 5' LATP2ΔICP4 fragment ($p<0.001$). Only the mid LATP2 segment and the full LATP2 (with and without the ICP4 region) appear to be enhancing expression from LAP1 at all at this time-point. Removing the ICP4 region from the 5' LATP2 and the full LATP2 does not increase expression in these viruses. If anything, in 1764/U_L43/3b, expression is reduced compared to that seen from the virus without this deletion in LATP2. If ICP4 were repressing at this site then at 3 days post-injection when immediate early proteins are present, deleting the region would be expected to result in an increase in expression, but the proposed reprieve from repression is not observed.

4.6.1.1.2 Series 3 viruses – LATP2 in the same orientation as LAP1

Figure 4-13, demonstrates the effect of the LATP2 deletions in the series 3 viruses. The virus with the mid LATP2 region (1764/U_L43/2c) is the only virus with a real difference in expression level of *lacZ* compared to the others. It gives barely any *lacZ* expression *in vivo* at 3 days post-injection. The *lacZ* expression is significantly lower than that from the 3' half of LATP2, full LATP2 and 5' half of LATP2ΔICP4-containing viruses ($p<0.01$). Comparing these results to the similar work of Berthomme *et al.* (Berthomme *et al.* 2001), there are some discrepancies. They found that a virus

containing either the whole LATP2 region or the 5' half gave much greater expression of *lacZ* than a virus either without LATP2, or with the 3' half. Again this is probably explained by the positioning of LATP2 with respect to LAP1 in the two different studies; in the viruses tested here LATP2 is upstream of LAP1, whereas the other group used it downstream of LAP1 in its natural position.

As with the series 2 viruses, removing the ICP4 region does not appear to have a great impact on the expression levels from the virus containing full LATP2. There is a very small increase in expression from the virus containing the 5' half LATP2 when the ICP4 region is removed but the fact that this is not also seen in the full LATP2 suggests that it is not due to alleviation of repression by ICP4.

4.6.1.1.3 The effect of orientation of LATP2 deletions on lacZ expression during lytic infection

Viruses containing the 3' half LATP2, the 5' half LATP2ΔICP4 and the full LATP2ΔICP4 show higher *lacZ* expression levels when in the same orientation as LAP1 than when in the opposite. They also are the only viruses to give higher levels of lacZ expression when in the same orientation as LAP1 than the virus lacking LATP2 (i.e. they show enhancer activity). The mid section of LATP2 only shows enhancer activity when in the reverse orientation to LAP1. This mid section of LATP2 is the only fragment that gives better enhancer activity than the whole LATP2, suggesting that one or more repressive elements have been deleted, probably from both ends of LATP2. This relief from repression is only seen when LATP2 is in the reverse orientation to LAP1, which is not usual for a classic enhancer, which by definition is bi-directional.

4.6.1.2 LATP2 activity 1 month post-injection

Results for *lacZ* expression at 1-month p.i. are shown in table 4-3 and figure 4-15. Selected representative ganglia are shown in figure 4-16a & b.

VIRUS	PALE NEURONS	DARK NEURONS	WEIGHTED TOTAL SCORE	S.E.M.
1764/UL43/1a (3' Half LATP2)	19.1	0.44	20	3.05
1764/UL43/2a (Mid LATP2)	14.4	0.44	13.8	2.22
1764/UL43/3a (5' Half LATP2)	6.8	0.4	7.6	1.78
1764/UL43/4a[1] (No LATP2)	8.3	0.4	9.1	1.38
1764/UL43/5a (Full LATP2)	11.1	0.3	11.7	1.66
1764/UL43/3b (5' LATP2ΔICP4)	2.9	0	2.9	0.81
1764/UL43/5b (Full LATP2ΔICP4)	8.2	0.1	8.4	1.15
1764/UL43/20.9[1] (Control)	26.6	1.3	29.2	17.4

VIRUS	PALE NEURONS	DARK NEURONS	WEIGHTED TOTAL SCORE	S.E.M.
1764/UL43/1c (3' Half LATP2)	15	0.3	15.6	2.73
1764/UL43/2c (Mid LATP2)	0.7	0	0.7	0.5
1764/UL43/3c (5' Half LATP2)	7.78	0.11	8	0.88
1764/UL43/4a[1] (No LATP2)	8.3	0.4	9.1	1.38
1764/UL43/5c (Full LATP2)	23	0.6	24.2	3.44
1764/UL43/3d (5' LATP2ΔICP4)	16.7	0.1	16.9	1.34
1764/UL43/5d (Full LATP2ΔICP4)	20.7	0.22	21.1	2.44
1764/UL43/20.9[1] (Control)	26.6	1.3	29.2	17.4

Table 4-3 **Averaged counts of *lacZ*-positive neurons 1 month p.i.**
LacZ positive neurons in ganglia L3 – L6 were recorded per animal, and scored according to whether staining for *lacZ* was pale or dark (n=10). Weighted total score = (# pale neurons + 2 times # of dark neurons)

[1] Data for 1764/UL43/4a and 1764/UL43/20.9 is the same for both series of viruses

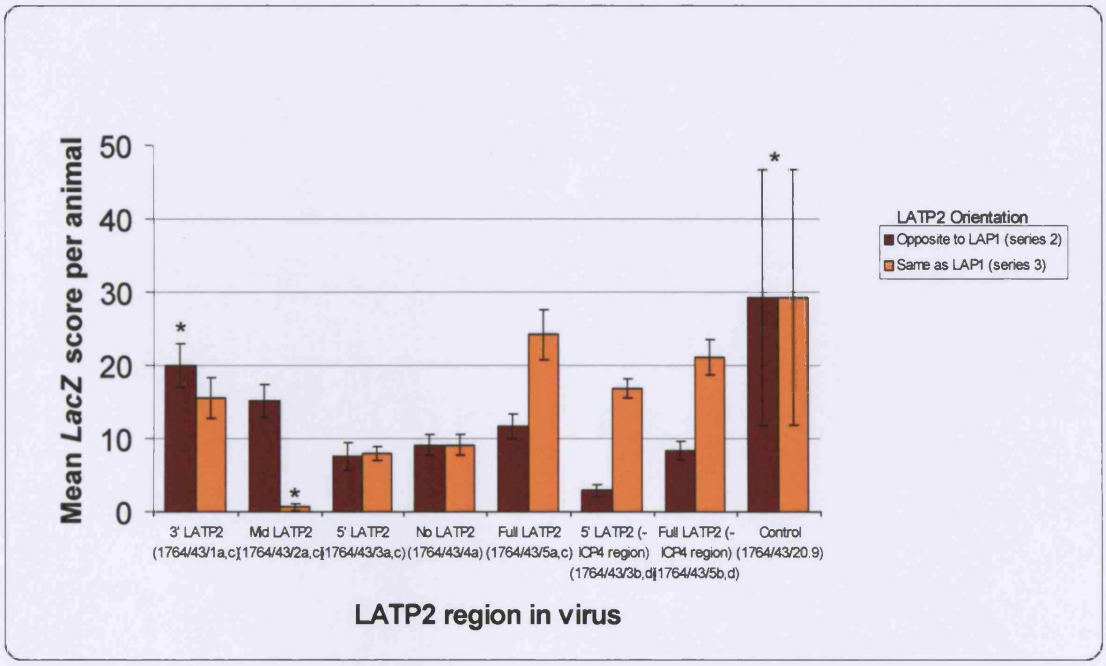


Figure 4-15 ***LacZ* expression from LATP2-deletion viruses 1 month post-injection in Balb/C SCID mice.**

Weighted *lacZ*-positive neuron scores give a semi-quantitative measure of *lacZ* expression from the LATP2 deletion viruses. (* - $p<0.05$)

These results show that the level of expression of *lacZ* at 1 month is lower (just over 2 times) than at 3 days for the series 2 and 3 viruses. This is not as large a drop as observed from the viruses in chapter 3, suggesting that it was the reduction in activity of the MMLV LTR between lytic and latent time-points that was responsible in that case.

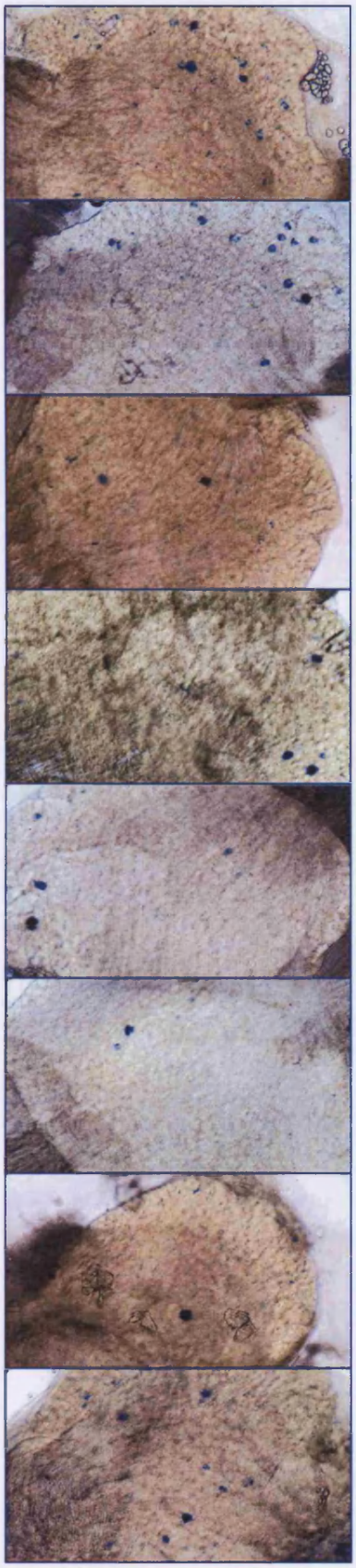


Figure 4-16a **Example ganglia 1 month post-injection showing *lacZ* expression – series 2 viruses.**

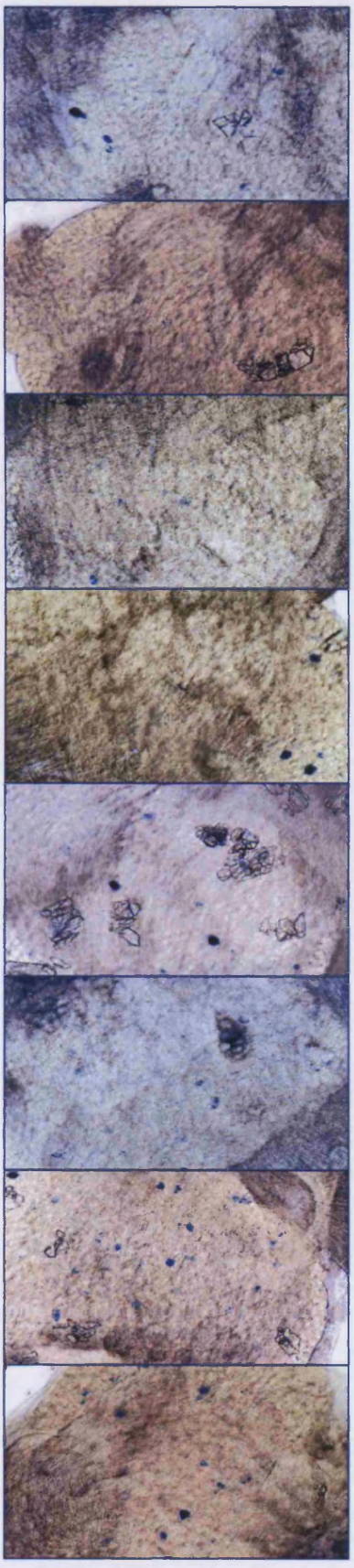


Figure 4-16b **Example ganglia 1 month post-injection showing *lacZ* expression – series 3 viruses.**

4.6.1.2.1 Series 2 viruses – LATP2 in the opposite orientation to LAP1

At 1 month p.i., when latency has been established, the viruses containing LATP2 fragments in the opposite orientation to LAP1 all give significantly less *lacZ* expression ($p<0.05$) than 1764/UL43/20.9, with the MMLV LTR present, except 1764/UL43/1a, containing the 3' half of LATP2. The virus without any part of LATP2 still gives some *lacZ* expression at 1 month from LAP1, which conflicts with the report of a similar virus in the literature (Berthomme *et al.* 2001), although others have found that there is low-level *lacZ* expression from LAP1 at latent times (Coffin *et al.* 1996b; Margolis *et al.* 1993). In fact there is very little difference in *lacZ* expression between the virus with no LATP2 and a full LATP2 region. The viruses with either the 3' half or the mid LATP2 allow slightly higher *lacZ* expression than the full LATP2. This points to an element that represses enhancer activity having been deleted in these two viruses, possibly in the 5' end not present in the mid LATP2 region.

4.6.1.2.2 Series 3 viruses – LATP2 in the same orientation as LAP1

Looking at the results in figure 4-15, the virus with the whole LATP2 region allows greater *lacZ* expression than any others (with or without the ICP4 region). The 5' half of LATP2 does not enhance expression from LAP1 alone and the mid-section of LATP2 causes reduced expression from the LAP1 promoter. This would suggest that an element required for long-term expression has been deleted from the mid section of LATP2, potentially in the region of DNA present only in the mid section but not the 5' half. These results conflict with those from Berthomme *et al.* (Berthomme *et al.* 2001) where they found latent transgene expression *in vivo* from viruses with either the full LATP2 or the 5' LATP2, but none from the 3' half of LATP2. These discrepancies once again can only be explained by the differences in arrangement of the LAT promoter/enhancer regions in the two studies.

Removing the ICP4 region from the full LATP2 has no effect on expression but removing it from the 5' half of LATP2 does increase expression slightly but not

significantly. As ICP4 does not play a significant role during latency, this increase must be due to some other effect of deleting this part. This was seen at 3 days as well in these viruses.

4.6.1.2.3 The effect of orientation of LATP2 deletions on long-term lacZ expression

Figure 4-15 demonstrates the effect of orientation of the LATP2 fragments in these viruses. The 3' LATP2 fragment and 5' LATP2 fragment allow a similar expression level of *lacZ* in either orientation, whereas the other fragments all differed in their ability to enhance *lacZ* expression, depending on which orientation they were in with respect to LAP1. Full LATP2 and the two ICP4 region deletion fragments drove more *lacZ* expression when in the same orientation as LAP1, whereas the mid-LATP2 fragment was only capable of driving *lacZ* expression when in the reverse orientation, which similarly to 3 days p.i. could be a result of an unusual enhancer activity.

4.7 DISCUSSION

This chapter aimed to characterise the enhancer and long-term expression functions of LATP2 in viruses containing deletion fragments of LATP2 in either orientation with respect to LAP1 driving *lacZ*. The deletion of the MMLV LTR and GFP from the viruses used in chapter 3 was carried out in the hope of clarifying the effect of LATP2 on LAP1.

Two sets of viruses were created with LATP2 in either orientation with respect to LAP1 driving *lacZ* in the UL43 locus within the 1764 viral backbone.

The different conditions under which the plasmids and viruses were tested gave differing results from the same fragments of LATP2. This is likely to be due to the variation in transcription and other factors depending on the cellular or viral environment as commented on previously. Also, different elements of the region are likely to be active at different times in the viral lifecycle. Therefore, general patterns of expression given by the different LATP2 fragments must be considered.

It has been shown previously that LATP2 has promoter, enhancer and long-term expression functions (Berthomme *et al.* 2000) and as such, assignment of elements within LATP2 to one function or another becomes complicated. Pure promoter activity from the LATP2 region should be studied without the presence of LAP1. The positioning of LATP2 with respect to LAP1 in the viruses tested in this chapter mean that promoter activity from LAP2 was not examined here.

Enhancer functions are complicated, with different mechanisms of action existing for different enhancers as discussed in section 1.2.2. By creating deletion mutants, positioning of various elements in relation to the promoter that they are acting on changes. This fact should be taken into account as well as the absence of DNA sequences.

Enhancers often work by the synergy of a number of different activators and repressors, many of which may localise to a relatively small area of DNA. Due to this, making large deletions as those in the above experiments may remove multiple elements of the enhancer and therefore perhaps give limited information about the make-up of the enhancer region.

Enhancers can also function in a structural manner such that they bind DNA-structure-altering proteins such as the HMG-I(Y) proteins that are known to bind to the LAP2 in this region (French *et al.* 1996). It has been shown that single point mutations that move or remove the binding site for these, for example in the mammalian interferon beta enhancer, disable the enhancer suggesting that an overall protein/DNA superstructure is critical (Thanos and Maniatis 1995). Therefore it is possible that the deletions of the LATP2 region made, alter the DNA structure of the region to a greater or lesser extent by removing sites for transcriptional modifiers that exert their action in this manner. This mechanism of action however results in bi-directional enhancer activity, which has not been universally seen in the experiments described above.

In the experiments described, LATP2 allowed greater expression of *lacZ* from LAP1 when in the same orientation than when in the opposite. No enhancer activity was seen from the 5' half of LATP2 in either orientation with respect to LAP1 *in vivo* or when in the opposite orientation *in vitro.* The mid section was able to direct as much enhancer activity as the whole LATP2 during lytic and latent infection when in the opposite direction to LAP1 but not in the same direction, which is unusual. The 3' half of LATP2 contains enhancer activity *in vitro* and *in vivo* during lytic infection when in the same orientation as LAP1 only and in both orientations at latent times. Therefore, it would seem that only the first 240bp of LATP2 does not contain any enhancer activity at all. This is in contrast to the similar work published concurrently (Berthomme *et al.* 2001) in which they found that enhancer activity was contained within the 5' half of the region only. This is probably explained by the differing arrangements of LAP1 and

LATP2 in the two studies, although enhancer activity ought to be the same irrespective of positioning or orientation with relation to a promoter. However, the enhancer contained within this region may not be conventional in terms of directionality as discussed below.

An insulator site could possibly explain the unidirectional enhancer activity exhibited by the mid-section of LATP2. As discussed in chapter 1 (section 1.2.3.5), insulators are found at boundaries of transcriptionally active/inactive regions of DNA and serve to protect neighbouring genes from unwanted transcription or repression. They often work by binding the CCCTC binding factor (CTCF) protein (Bell *et al.* 1999). If an insulator site has been created in virus 1764/U_L43/2c (mid LATP2 in the same direction as LAP1) at the junction between LAP1 and mid LATP2, then this could explain the unusual activity seen. Analysis by BLAST search of the sequence for the CTCF binding sequence CCCTC at this junction did not show any matches, however it is possible that a previously unidentified, different type of insulator site has been created. Alternatively, a repressive element may exist in the mid section of LATP2 that only functions in one direction.

The finding that when the 5' half of LATP2 is removed from the plasmids or viruses studied here, an increase in expression from that given by the full LATP2 region is observed in neuronal cells *in vitro* or *in vivo* could suggest the presence of a neuronal repressive element in the 5' half of LATP2.

It is possible therefore that repressive elements are located in the region of LATP2 common to both the 5' half and mid section. This is a 400bp stretch of DNA containing several putative transcriptional regulatory elements as discovered in chapter 3 using the TFSEARCH program (Heinemeyer *et al.* 1998) including binding sites for the potentially repressive factors MZF1 and ZID.

Removing the ICP4 responsive region from the 5' half or full LATP2 had little effect on expression levels; the only time any effect was seen was in the transient transfection of the plasmids where expression was increased when full or 5' LATP2ΔICP4 (depending on cell type) was in the same orientation as LAP1. However, ICP4 is not present in the transient expression experiments and the increase in expression observed there is probably due to removal of a transcriptionally repressive element found at the same position. The original reporting of repression within this region by ICP4 was on promoter activity of LAP2 (Goins *et al.* 1994), which is quite distinct from the enhancer activity seen in these studies, therefore it appears that ICP4 does not affect the enhancer activity within this region.

For long-term transgene expression, the 3' half of LATP2, or part thereof seems to be required in either orientation. In the experiments described above, removing the 3' half of LATP2 resulted in the same level of *lacZ* expression as that from LAP1 alone at a latent time *in vivo*, i.e. the 5' half of LATP2 is not sufficient for increased long-term expression from LAP1. This is in contrast to the work published during these experiments (Berthomme *et al.* 2001) where they found that the 5' half of the region was sufficient for long-term expression and that the 3' half alone was not. As stated previously these differences are probably due to the slightly different arrangement of promoters in the two studies.

A summary diagram of the LATP2 region showing the location of potential enhancer and long-term expression elements as well as repressive elements as suggested by the experimental data is shown below (figure 4-17).

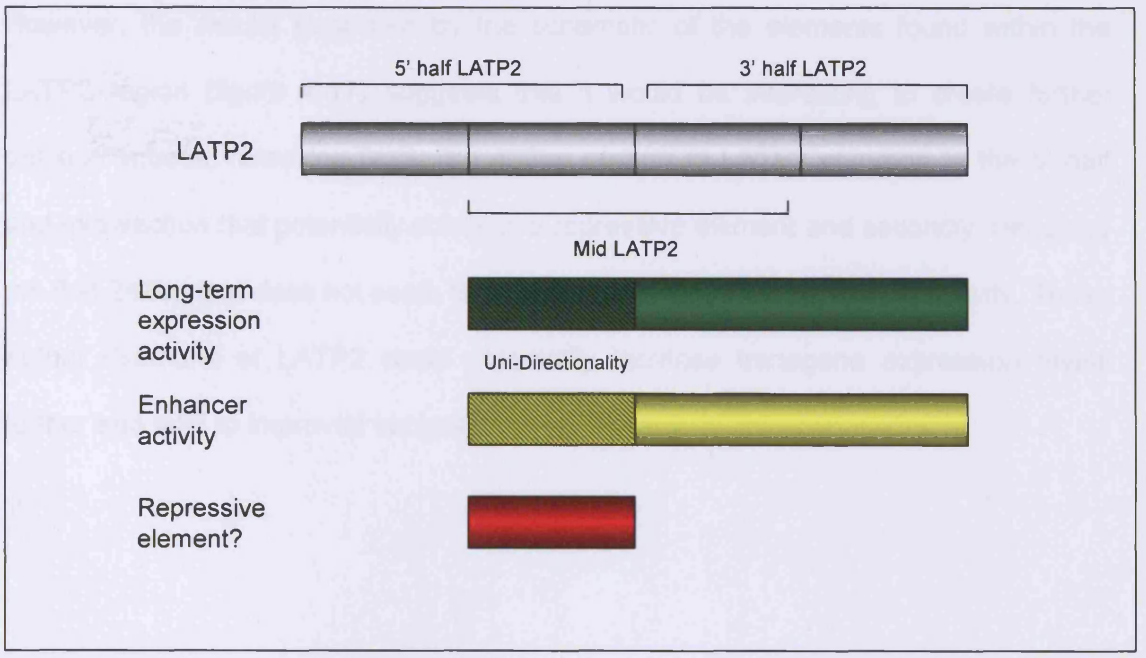


Figure 4-17 Schematic illustration of the proposed location of enhancer and long-term expression functions within LATP2

The hatched region shows enhancer or long-term activity that derives from the section when in one orientation only. The un-hatched areas show bi-directional activity.

This is no doubt an over-simplified model of LATP2 function, with possibly numerous activators and repressors of transcription working at different times in the viral lifecycle to modulate lytic infection and latency. The suggestion of these many active and repressive elements throughout LATP2 perhaps shows that the contribution of the region to LAT production in wild-type virus is tightly regulated in both lytic and latent infection.

The fact that the experiments described here show that maximum transgene expression in these vectors is provided when the full LATP2 region is in its natural orientation with respect to LAP1 during latency, suggests that elements located throughout the region are likely to be required for optimal long-term expression.

However, the results illustrated by the schematic of the elements found within the LATP2 region (figure 4-17) suggests that it would be interesting to create further deletion viruses, removing firstly the 400bp section of LATP2 common to the 5' half and mid-section that potentially contains a repressive element and secondly, removing the first 242bp that does not seem to have any positive effect on LATP2 activity. These further deletions of LATP2 could potentially increase transgene expression levels further and lead to improved vectors for gene therapy.

CHAPTER 5:

OPTIMISATION AND APPLICATION OF THE CHROMATIN IMMUNOPRECIPITATION ASSAY FOR STUDYING THE ACETYLATION OF THE GENOME OF HSV1

5.1 INTRODUCTION

As discussed in section 1.2.3.2, a common method of transcriptional control is via histone modification; either by phosphorylation, methylation, acetylation, or occasionally ubiquitination. Acetylated histones are a marker of transcriptionally permissive euchromatin, whilst methylated and phosphorylated histones are associated with a more tightly packed, transcriptionally silent heterochromatin (Bradbury *et al.* 1973; Hebbes *et al.* 1988; Kleinschmidt and Martinson 1981; Marushige 1976; Rea *et al.* 2000). It is often the promoter and/or enhancer region of a gene that becomes associated with acetylated histones when it is active or about to be active (Kuo *et al.* 1998).

Evidence that modification of histones is involved in the control of transcription of herpes viruses has slowly appeared over time. It is known that the HSV-1 genome is associated with nucleosomes during latency in a chromatin-like structure (Deshmane and Fraser 1989). The LAP2 promoter contained within LATP2, can directly bind a protein similar to the group of small chromosomal chromatin-associated proteins HMG I(Y) (high motility group) (French *et al.* 1996). HMG proteins regulate transcription as part of an enhanceosome by binding to the DNA and altering the DNA configuration (Falvo *et al.* 1995; Grosschedl *et al.* 1994). Specifically, in LAP2, HMG I(Y) appears to facilitate the binding of transcription factor Sp1. The same HMG 1 was also found to act as a co-activator of ICP4 expression (Carrozza and DeLuca 1998).

Perhaps the most convincing report of the involvement of histone modification in modulation of herpesvirus gene expression is that the genome of Epstein-Barr virus is arranged on nucleosomes (Dyson and Farrell 1985) and the promoter of the viral immediate-early gene BZLF1 is associated with acetylated histone H4 on induction of reactivation from latency *in vitro* (Jenkins *et al.* 2000).

At the start of this project, the only evidence for histone modification as a transcriptional control mechanism for HSV1 was that described above and some emerging work that ICP0 (both that of HSV1 and Bovine herpesvirus-1) had been found to interact with histone de-acetylases (HDACs) which was predicted to perhaps be a control mechanism used in the regulation of latency and reactivation (Hobbs and DeLuca 1999; Lomonte *et al.* 2001; Tsavachidou *et al.* 2001; Zhang and Jones 2001). Furthermore it was found that the inhibitor of HDACs, TSA, caused reactivation of HSV1 from neuronal cultures (Arthur *et al.* 2001).

The search performed to identify potential TF binding sites in LATP2 described in chapter 3 (section 3.2) identified a site for p300 within the 3' half of LATP2. p300 possesses histone acetyl transferase (HAT) activity (Ogryzko *et al.* 1996) and as such can help alter the DNA conformation to be more transcriptionally permissive. p300 interestingly acetylates HMG I(Y) *in vitro* but this has a negative effect caused by disruption of the enhanceosome of which HMG I(Y) is a component (Munshi *et al.* 1998).

This all led to the idea that studying the acetylation status of histones at HSV1 LAT and non-LAT promoters at different times in the viral life-cycle may elucidate a mechanism by which the latency promoters stay active, the hypothesis being that the LAT region chromatin is differently acetylated as compared to the rest of the genome during latency and thus allow a more open chromatin configuration which allows continued transcription. It is thought that enhancers may regulate transcription through the acetylation of histones (Agalioti *et al.* 2000), (see section 1.2.3.2), so whether particular elements of the LATP2 region are responsible for this phenomenon, if observed, would be explored, as would whether they correspond with the active/repressive regions identified in the previous chapters.

As discussed previously, it has been shown that LATP2 is capable of maintaining expression from exogenous promoters during latency when placed next to the region in a LAT or non-LAT locus (Lilley *et al.* 2001; Palmer *et al.* 2000). To investigate whether acetylation of associated histones is a mechanism by which this is achieved, it was decided that as well as looking at the acetylation status of promoters in the HSV1 genome, the acetylation status of an exogenous promoter would be studied when linked to LATP2 and when separate from LATP2.

5.1.1 *In vitro* models of HSV1 latency.

An *in vitro* system was chosen to be set up in order to study the acetylation of these viral genomes both during the lytic cycle and during latency, as it was considered advantageous to be able to work with a higher quantity of virus than would be available from *in vivo* models. The use of *in vitro* models to study latency of HSV has been somewhat controversial over the years, with their relevance to the *in vivo* situation being debated. Three different types of *in vitro* model have been described, with differing attributes:

Primary cultures of neurons have been favoured as representing most closely the *in vivo* situation and used by a number of groups to establish an NGF-dependent infection (Kennedy *et al.* 1983; Wilcox and Johnson, Jr. 1987; Wilcox and Johnson, Jr. 1988). They have been shown to allow reactivation on withdrawal of NGF and express LATs (Doerig *et al.* 1991; Smith *et al.* 1994) but not lytic cycle genes during latency. They do typically require viral replication to be limited either by antivirals or replication-defective mutants in order to allow a latent state. However, in the absence of ICP0, establishment of latency is greatly reduced, which is not seen *in vivo* (Wilcox and Johnson, Jr. 1987). These are troublesome to prepare and still a large amount of tissue is required to obtain reasonable numbers of neurons for study.

Infected fibroblasts present perhaps a more contentious model of latency. They were first used by Wigdahl *et al* (Wigdahl *et al.* 1981; Wigdahl *et al.* 1982) in the form of human embryo lung cells. It was shown that only limited gene expression from HSV1 is required to establish latency in fibroblasts and that ICP0 is not necessary. However, ICP0 is required and sufficient for reactivation (Russell *et al.* 1987). The HSV genome takes on a non-linear form as during *in vivo* latency (Harris and Preston 1991) but the fibroblasts do not allow the production of LATs, which is perhaps the biggest problem with this model, meaning that HSV is in a 'quiescent' state rather than a latent one, with the whole of the genome shut down.

The final *in vitro* model that has been used is that of infected neuronally derived cells. PC12s are from a rat adrenal pheochromocytoma and differentiate into cells resembling sympathetic neurons after treatment with NGF (Greene and Tischler 1976). They were first used for infection with HSV1 by Rubenstein and Price (Rubenstein and Price 1983) and have been shown to support high-levels of LAT production during lytic and latent infection (Bratanich and Jones 1992; Danaher *et al.* 1999; Leib *et al.* 1991). However it is the 2kB LAT rather than the 1.5kB species found *in vivo* that is produced and furthermore, this LAT production requires viral replication to have occurred – ICP0 mutants were found not to produce LATs (Rodahl and Haarr 1997). HSV-infected PC12 cells cannot be reactivated by HSV1 super-infection as is possible in fibroblasts (Su *et al.* 2000).

ND7s are potentially the most useful cell line to create a similar latent model to that *in vivo*. They are a fusion of immortalized primary DRGs and a neuroblastoma cell line and have the properties of nociceptive sensory neurons (Wheatley *et al.* 1990; Wood *et al.* 1990). Both 1.5Kb and 2Kb LATs are transcribed in infected cells whilst the IE genes are not (Mador *et al.* 1995). A neuronal specific inhibitory factor binds to the TAATGARAT motif in the viral IE promoters and prevents transactivation (Wheatley *et al.* 1991).

5.1.2 Choice of viruses for analyses

The backbone of the viruses was chosen as one that is disabled and replication incompetent. It has been shown that HSV1 viruses that do not express any IE genes are pushed towards the latent pathway and enter the quiescent state *in vitro* (Harris and Preston 1991; Preston and Nicholl 1997). Therefore the backbone of the viruses used have the 2 essential genes for ICP4 and ICP27 deleted and prevent transactivation of the remaining IE genes by including a disabling mutation in the gene for the virion protein VP16 (Lilley *et al.* 2001). The same viruses can be used to study acetylation at lytic timepoints by infecting a complementing cell line with the virus, in this case the cell line 27/12/M:4 which expresses ICP4 and ICP27 (Thomas *et al.* 1999b). The VP16 mutation can be complemented by the addition of HMBA to the growth media (McFarlane *et al.* 1992).

Although it would have been consistent to study the pR20.9 cassette as used in chapter 3, with the MMLV LTR as the exogenous promoter linked to LATP2; as previously discussed, MMLV has been shown to have its own long-term expression capabilities and if these function in a similar way to those of LATP2, this could confuse the issue of whether any acetylation seen associated with MMLV was due to its positioning adjacent to LATP2 or due to its own characteristics. Therefore the cassettes chosen to be studied were one in which the non-HSV1 IE promoter/enhancer CMV, driving GFP was placed next to LATP2 in the LAT region (pR19GFP) and the second where the same CMV promoter driving GFP was positioned in a non-LAT locus, not linked to LATP2 (CMVGFP/US5). GFP expression from this virus does not occur during latency (unpublished observations), as CMV does not have long-term expression capabilities. Furthermore, a virus in which the CMV promoter was placed next to the LATP2 region has previously been shown to allow transgene expression during latency, whereas when the promoter was placed next to LAP1 only short-term expression was given (Palmer *et al.* 2000).

These two viruses also allow the simultaneous study of the acetylation profile of LAT and non-LAT promoters. Figure 5-1 shows the structure of these viruses.

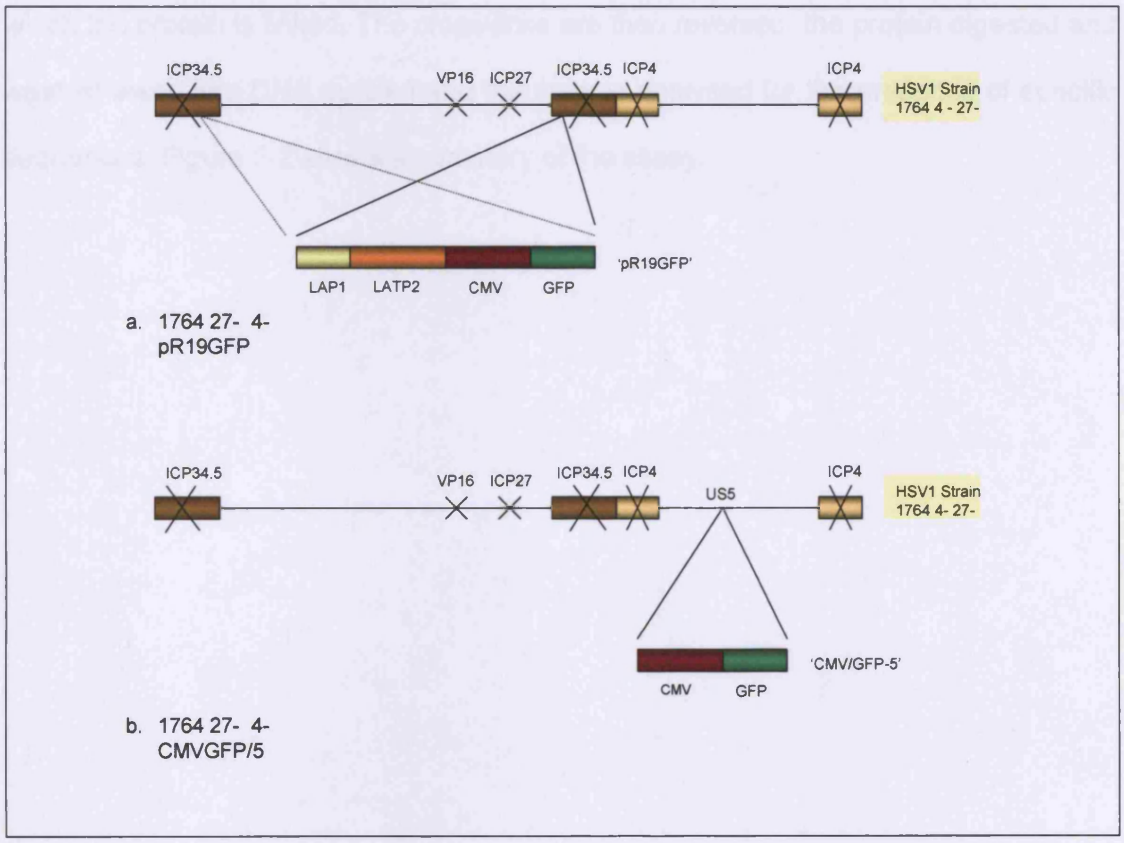


Figure 5-1 Schematic diagram showing the structures of the two viruses used in the acetylation study.

a. The CMV promoter is placed next to the LATP2 promoter in the LAT region. This therefore is present in two copies per virus.
b. The CMV promoter is inserted into the US5 region and is therefore present in only one copy per virus.

5.1.3 The chromatin immunoprecipitation (ChIP) assay

The chromatin immunoprecipitation (ChIP) assay is the most precise method currently available to identify specific proteins associated with a region of DNA. It has been refined since the first use in the 1980s (Gilmour and Lis 1985). The basis of the assay is that associated proteins are cross-linked to the DNA with formaldehyde; the chromatin is sheared or digested into smaller fragments and then the protein of interest immunoprecipitated using an antibody. This also brings down the DNA to which the protein is linked. The cross-links are then reversed, the protein digested and washed away, the DNA purified and the sample analysed for the presence of specific sequences. Figure 5-2 shows a summary of the assay.

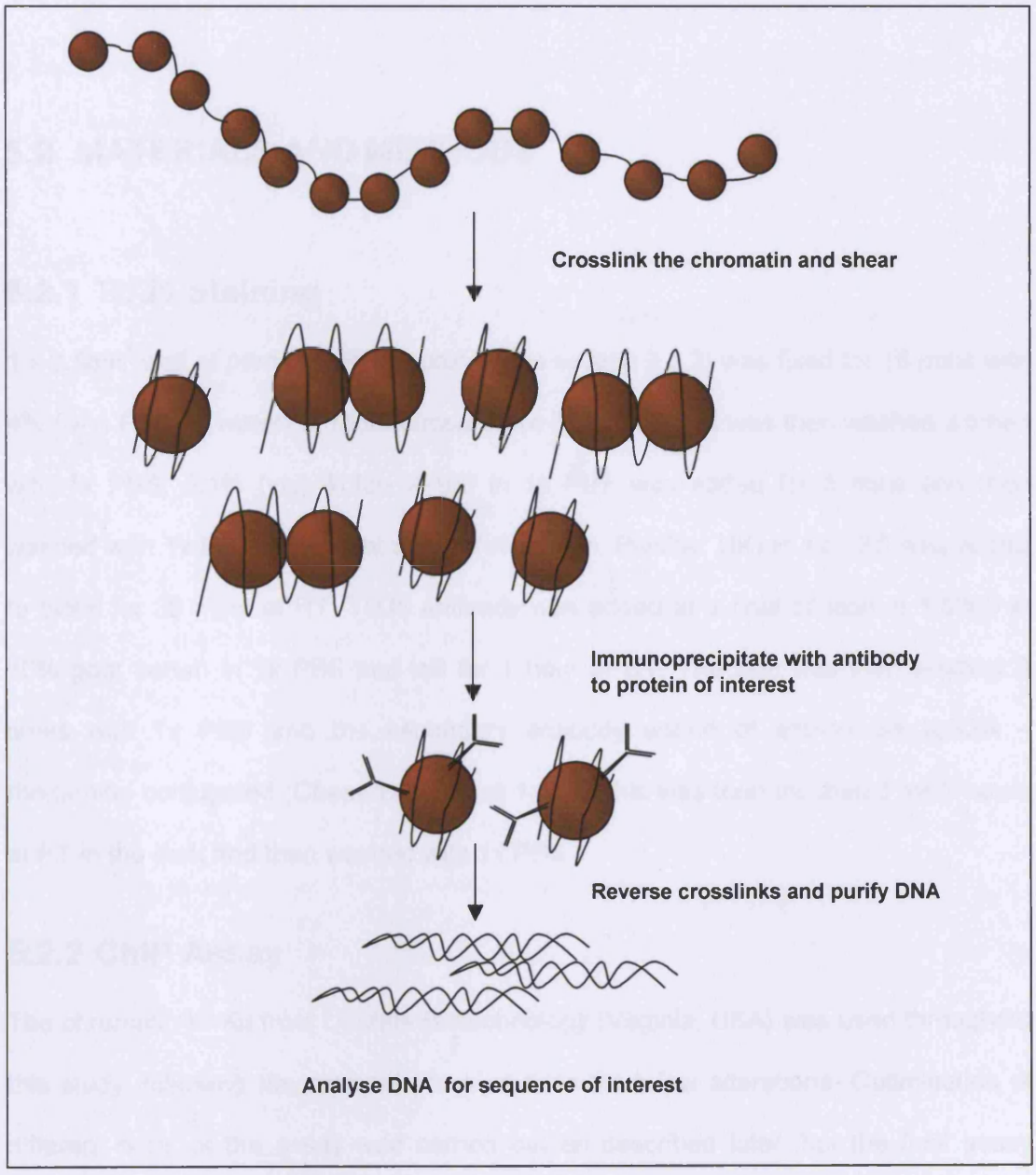


Figure 5-2 Basic illustration of the ChIP assay

At the start of this study, there was no literature on the use of the ChIP assay with HSV1 infected cells or tissue. Therefore, the method had to be optimised in conjunction with the *in vitro* systems to be used.

5.2 MATERIALS AND METHODS

5.2.1 TUJ1 Staining

1 x 3.5cm^2 well of primary DRG neurons (see section 2.3.3) was fixed for 15 mins with 4% (w/v) PFA (in water) at room temperature (RT). The well was then washed 3 times with 1x PBS. 0.1% (v/v) Triton X-100 in 1x PBS was added for 5 mins and then washed with 1x PBS. 20% goat serum (Invitrogen, Paisley, UK) in 1x PBS was added to block for 30 mins at RT. TUJ1 antibody was added at a final dilution of 1:5000 in 10% goat serum in 1x PBS and left for 1 hour at RT. The well was then washed 3 times with 1x PBS and the secondary antibody added of anti-mouse IgG2A – rhodamine conjugated (Chemicon, UK) at 1:100. This was then incubated for 2 hours at RT in the dark and then washed with 1x PBS.

5.2.2 ChIP Assay

The chromatin IP Kit from Upstate Biotechnology (Virginia, USA) was used throughout this study, following the protocol provided but with a few alterations. Optimisation of different parts of the assay was carried out as described later, but the final assay protocol used is described here.

5.2.2.1 Crosslinking and sonication

Histones were cross-linked to DNA by adding 37% Formaldehyde (Sigma) to the culture medium to a final concentration of 1% and incubated at 37°C for 10 mins.

The media was removed and the cells washed twice in ice cold 1x PBS containing a cocktail of protease inhibitors (Roche, E. Sussex, UK). Cells were scraped into a 1.5mL tube and pelleted at 4000rpm for 4 mins at 4°C in a 5417R microfuge (Eppendorf, Germany). The cell pellet was resuspended in 200μL of warmed SDS lysis buffer containing protease inhibitors and incubated on ice for 10 mins. 400μL of ChIP dilution buffer was added, containing protease inhibitors and the sample was sonicated in a cold room (4°C) for 4 bursts of 40 seconds, with 1 min rest on ice in between using a GE50 ultra sonic processor (Jencons, Beds., UK) set to 30% power. 5μL of each sample was removed in order to check for the size of sonicated fragments. These were run on a 1.2% agarose gel and visualised under UV light. If the majority of the smear was <600bp then sonication was deemed to be efficient. The sonicated sample was centrifuged for 10 mins at 4°C at 13000rpm and the supernatant (s/n) transferred to a new tube. The s/n was then diluted with ChIP dilution buffer containing protease inhibitors so that the final volume of each sample was 2mL. 50μL of each sample was then removed and stored at -20°C until ready to reverse the crosslinks. This is the input sample.

5.2.2.2 Immunoprecipitation

The samples were then pre-cleared with 80μL of salmon sperm DNA/Protein A agarose-50% slurry for 30 mins at 4°C on a rotating wheel. The agarose was then pelleted by pulse spinning the sample and carefully removing the supernatant to a new tube. The immunoprecipitating antibody to acetylated histone H3 (K9,14) (Upstate, Virginia, USA) was then added at 5μL/mL to the sample and left overnight at 4°C on a

rotating wheel. A control sample was always included in which an anti-IgG antibody (Sigma) was added at a concentration of 1μL/mL.

5.2.2.3 Washing and reversal of cross-links

60μL of salmon sperm DNA/protein A agarose slurry was added to each sample and left for 1 hour at 4°C on a rotating wheel to collect the antibody/histone complex.

The agarose was pelleted by pulse-spinning the samples for 7 seconds and the s/n that contained the unbound fraction was discarded. The pellet containing the agarose/antibody/histone was carefully washed for 4 mins each on a rotating platform with 1mL of each of the buffers in order: 1) Low salt immune complex wash buffer; 2) High salt immune complex wash buffer; 3) LiCl immune complex wash buffer; 4) 1x TE – two washes. The histone complex was then eluted from the antibody by adding 275μL fresh elution buffer (1% SDS, 0.1M $NaHCO_3$, (Sigma)) and incubating at RT for 15 mins with rotation. The agarose was pulse-spun and 200μL of s/n carefully transferred to a new tube. The elution was repeated with 250μL elution buffer and after incubation 300μL removed to combine with the other aliquot of eluent. Reversal of cross-links was carried out by adding 5M NaCl (VWR) to each sample, including the input sample removed previously and incubating for 4 hours at 65°C.

5.2.2.4 Clean-up and purification

RNase A (sigma) was added to the samples at a final concentration of 40μg/mL, vortexed and then incubated for 30 mins at 37°C. Protein was degraded by adding EDTA to a final concentration of 0.01M, Tris pH 6.5 to a final concentration of 0.02M and proteinase K (Roche, E. Sussex, UK) to a final concentration of 40μg/mL. The samples were vortexed and incubated for 45°C for 1 hour.

Purification of the DNA was carried out using a QIAquick PCR purification kit (Qiagen, W. Sussex, UK), according to the manufacturer's instructions and adding 3M sodium

acetate (sigma), pH 6.5 to ensure that the pH of each sample was pH 7 or less before applying to the columns.

5.2.3 PCR

Where standard PCR was used, the GC rich kit (Roche, E. Sussex, UK) was used as described in section 2.2.4.2 due to the GC rich nature of HSV1 DNA.

5.2.4 Quantitative PCR

Quantitative PCR was carried out using SYBR Green from the QuantiTect SYBR Green PCR Kit (Qiagen, W. Sussex, UK) as per the manufacturers instructions. Primers for DNA regions of interest were used at 0.3μM final concentration each. An ABI 7000 Sequence Detection System (Applied Biosystems, California, USA) was used to run the PCR with the following program:

50°C	2 mins	x1cycle
95°C	10 mins	x1cycle
95°C	15 secs	} x40 cycles
60°C	1 min	

Melting curves were examined for each set of primers used to ensure that the products were specific.

5.2.5 Nuclei Isolation

Nuclei isolation was carried out with the Nuclei EZ Prep Nuclei Isolation Kit (Sigma, Dorset, UK). Briefly, cells were grown on 90mm plates and infected as required. Cells were washed with 10mL of ice cold PBS, then harvested and lysed by adding 4mL of

Nuclei EZ lysis buffer, centrifuging at 500g for 5 mins, 4°C and removing the s/n. The nuclei pellet was washed with 4mL of lysis buffer, centrifuged as before and the pellet placed on ice. The nuclei were resuspended in 200μL of ice-cold Nuclei EZ storage buffer and used immediately.

5.3 OPTIMISATION OF THE *IN VITRO* MODELS

5.3.1 Lytic model

The lytic model chosen was the use of 27/12/M:4 cells as described above they complement for the proteins ICP4 and ICP27. HMBA was always included in the growth medium to counteract the mutation in VP16. As there was no alternative to this cell line then this set-up did not need much optimisation as it was well established in the laboratory. An infection at an MOI of 1 had been shown previously to be efficient at delivering virus to all cells. Figure 5-3 shows the infections at 1 and 2 days post-infection with the virus 1764 4- 27- pR19GFP. It was initially chosen to look at acetylation of the virus in these cells after 1 day, representing a lytic infection.

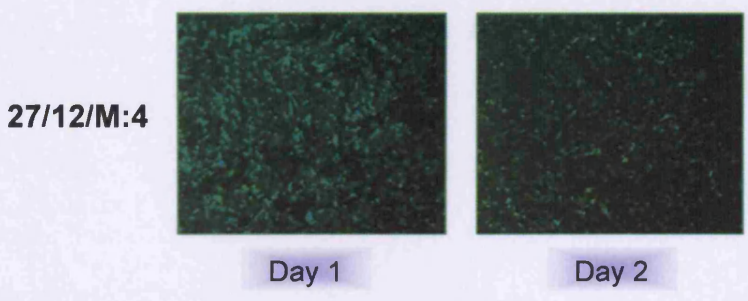


Figure 5-3 1764 4- 27-pR19GFP virus in the complementing cell line 27/12/M:4

Cells were infected an MOI of 1 in serum free media. After 1-hour full growth media was added containing HMBA.

5.3.2 Latent model

To establish which cell line would be most appropriate for the latent model a number of different non-complementing cell lines available in the lab were infected and left for up to 6 days – a period of time far in excess of that required for a replication incompetent virus to become quiescent to ensure their suitability for the assay. The infections were assessed for infectability and stability throughout the experiment. The cell lines used were BHKs, Veros, HT1080s and ND7s. They were all infected an MOI of 5 with the virus 1764 4- 27- pR19GFP. This virus had been filtered to ensure that no viral proteins were carried over from the 27/12/M:4 complementing cells that they were grown on. Figure 5-4 shows the infected cell lines at different times post-infection.

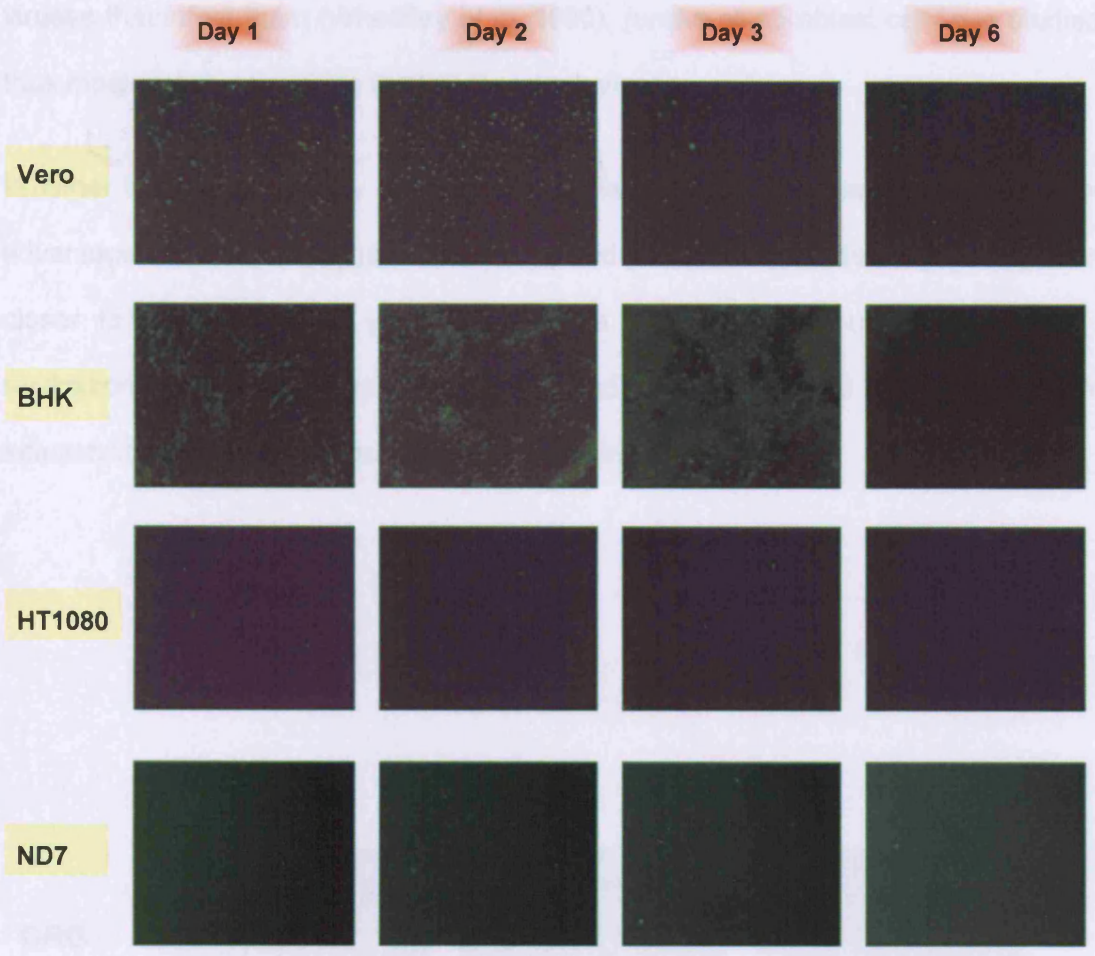


Figure 5-4 1764 4- 27-pR19GFP virus in a panel of non-complementing cell lines.

Cells were infected an MOI of 5 in serum-free-media. After 1-hour full growth media was added. The media was changed every two days during the period of infection.

From figure 5-4 it can be seen that BHK cells were the most efficiently infected with the replication incompetent virus, followed by the Vero cells and then the ND7 cells. However, the BHK cells were looking very unhealthy by 2 days post-infection. The ND7 cells looked the healthiest at the end of the experiment and also appeared to show an increase in GFP expression from the virus between 1 and 2 days post-infection, suggesting that latency may be being established. Therefore these cells were deemed to be the most suitable for the *in vitro* assay. These are also the most appropriate as ND7 cells have been shown to allow the production of LATs from

viruses that infect them (Wheatley *et al.* 1990), (unlike all fibroblast cell lines studied) thus most closely mimicking the latent state *in vivo*.

Whether the use of primary dorsal root ganglia cultures for the latent model may be advantageous was investigated. As described above, this model is probably even closer to the situation *in vivo*. Therefore, a culture was set-up, infected with a replication incompetent virus at a MOI of 5 and maintained for 10 days to assess the infectability and stability. Figure 5-5 shows the infected cultures.

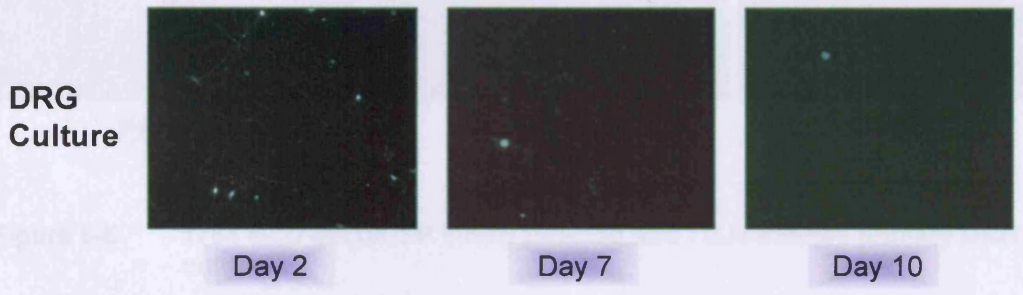


Figure 5-5 **1764 4- 27-pR19GFP virus in a 1° DRG Culture**

Cells were infected an MOI of 5 in serum-free-media. After 1-hour full growth media was added. The media was changed every two days during the period of infection.

The infected DRG cultures looked healthy up to at least 10 days.

To show that the cells being infected were in fact neurons and not supporting cells, a new infection was set up and after 2 days they were processed for immunofluorescence with the anti-neuronal specific β-tubulin antibody TUJ1 as in section 5.2.1. Figure 5-6 shows that when the staining is compared to the GFP infection at 2 days it can be seen that indeed the same neurons as stain positive for TUJ1 are infected.

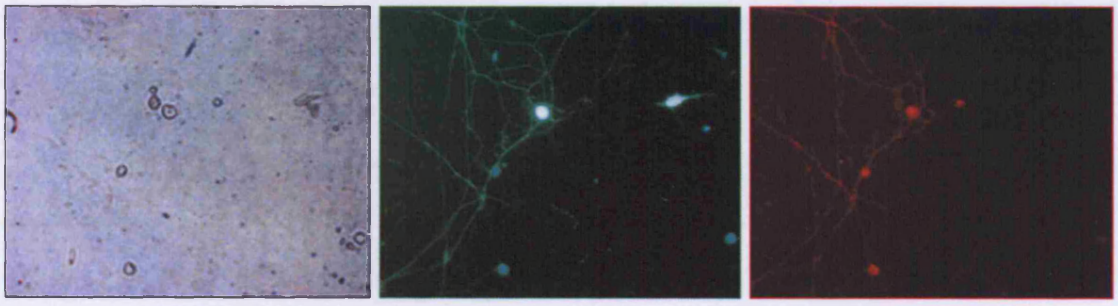


Figure 5-6 **1764 4- 27-pR19GFP virally infected and TUJ1-stained primary DRG culture.**

Adult mouse primary DRG cultures were infected with 1764 4- 27- pR19GFP and 2 days p.i. photographed for GFP expression before processing for immunofluorescence and photographing the same frame.

5.3.3 Production of LATs *in vitro*

As stated above, infected ND7 cells allow production of LATs. To check that the replication-incompetent virus 1764 4- 27- still produced LATs *in vitro*, the RNA at different times post-infection was examined. ND7s were infected at an MOI of 5 with 1764 4- 27- CMV/GFP/5, the RNA extracted at 16, 24, 48 and 72 hours post-infection

and cDNA made (see section 2.2.5). To compare the production of LATs in primary DRG cultures, RNA was also extracted from a similarly infected culture at 4, 7 and 10 days post-infection. PCR was carried out using the primers below for the cellular gene cyclophilin A, the HSV1 LAT region and the IE gene for ICP22. Figure 5-7 shows the results of the PCRs. (Only ICP22 and LAT primers were used for the 1° DRG culture).

GENE	FORWARD PRIMER	REVERSE PRIMER
CYCLOPHILIN	CGAGCTGTTTGCAGACAAAG	TTCTTGCTGGTCTTGCCATT
ICP22	GCTTCCTTGTTTGGAGACCA	GTCCAGTCAAACTCCCCAAA
LAT	GACAGCAAAAATCCCCTGAG	ACGAGGGAAAACAATAAGGG

Table 5-1 Primers used in RT PCR reactions

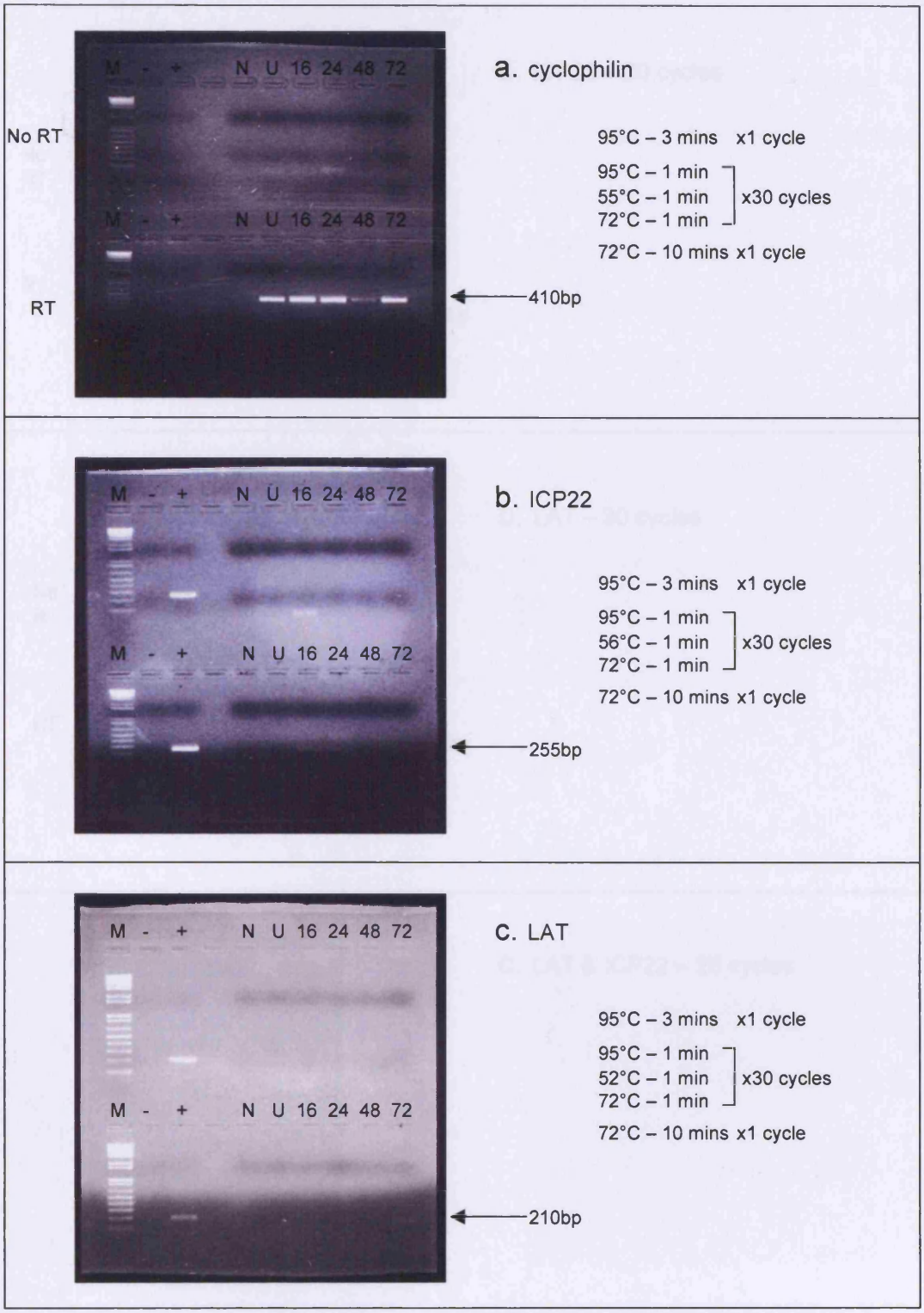


Figure 5-7 PCR on cDNA made from RNA of ND7 cells infected with 1764 4- 27-CMVGFP/5

a. With primers for the cellular gene cyclophilin A
b. With primers for the IE gene ICP22
c. With primers for the LAT gene

M= 1Kb+ DNA Marker, - and + are PCR controls, N = No sample RT, U = uninfected cells, 16, 24, 48 & 72 denote hours post-infection.

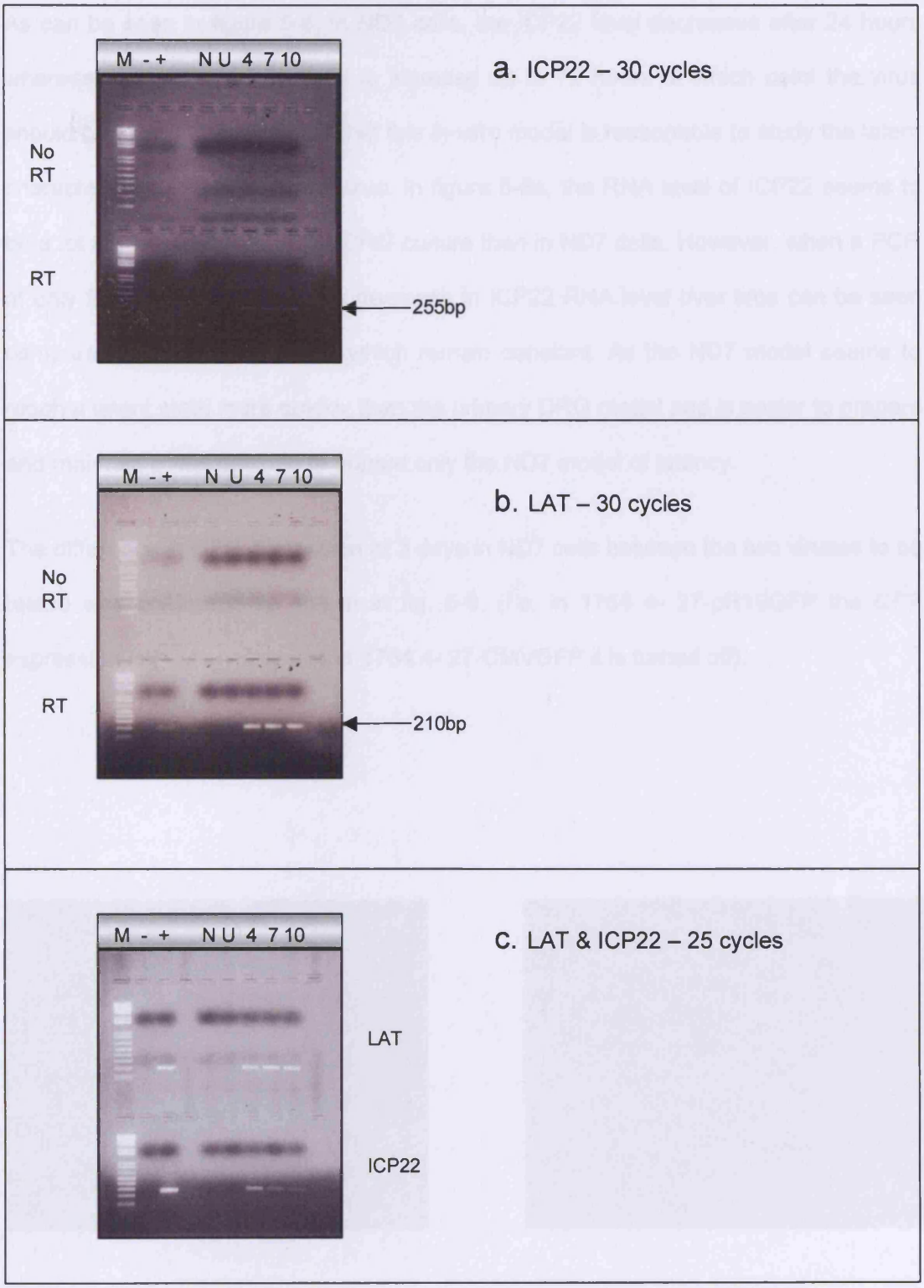


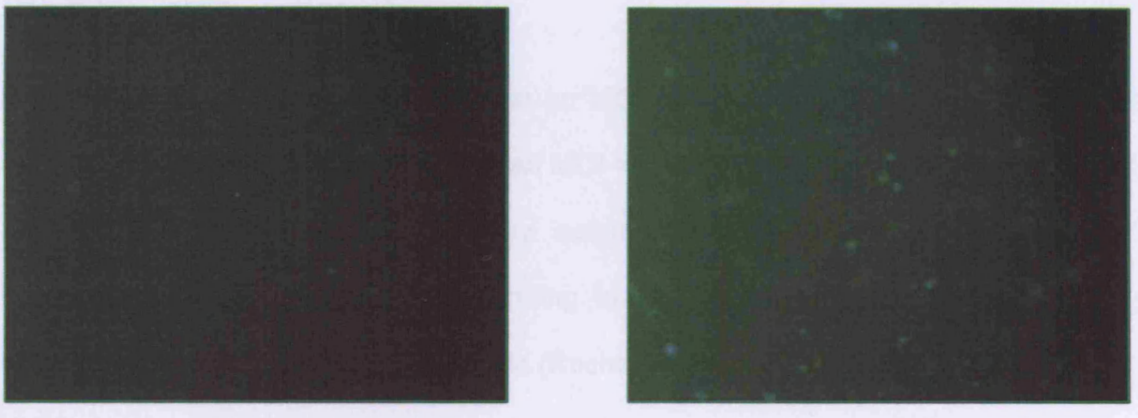
Figure 5-8 **PCR on cDNA made from RNA of a primary DRG culture infected with 1764 4- 27- CMVGFP/5**

a. With primers for the IE gene ICP22 – 30 cycles of PCR
b. With primers for the LAT gene – 30 cycles of PCR
c. With primers for both LAT & ICP22 – 25 cycles of PCR

M= 1Kb+ DNA Marker, - and + are PCR controls, N = No sample RT, U = uninfected cells, 4, 7 & 10 are days post-infection.

As can be seen in figure 5-8, in ND7 cells, the ICP22 level decreases after 24 hours whereas the LAT level appears to increase up to 72 hours at which point the virus should be latent. This confirms that this *in vitro* model is reasonable to study the latent characteristics of this disabled virus. In figure 5-8a, the RNA level of ICP22 seems to be a lot higher for longer in the DRG culture than in ND7 cells. However, when a PCR of only 25 cycles was used, the decrease in ICP22 RNA level over time can be seen compared to that of the LATs, which remain constant. As the ND7 model seems to reach a latent state more quickly than the primary DRG model and is easier to prepare and maintain it was decided to pursue only the ND7 model of latency.

The difference in GFP expression at 3 days in ND7 cells between the two viruses to be tested was confirmed as shown in fig. 5-9. (I.e. in 1764 4- 27-pR19GFP the GFP expression is kept on whereas in 1764 4- 27-CMVGFP it is turned off).



1764 4- 27-CMVGFP/5 **1764 4- 27-pR19GFP**

Figure 5-9 ND7 cells infected with 1764 4- 27-pR19GFP and 1764 4- 27- CMVGFP/5 after 3 days.

5.3.4 Nucleosomal structure of the virus *in vitro*

Several groups have previously tested the structure of HSV1 DNA by micrococcal nuclease digestion. It was first carried out on cells infected with wild-type HSV1; one group observed no nucleosomal units on digestion (Mouttet *et al.* 1979), whilst another observed only a small fraction (Leinbach and Summers 1980). It was subsequently carried out on cell preparations from lytically infected brainstems of mice upon which a nucleosome-like fraction was detected (Muggeridge and Fraser 1986). When latently infected brainstems of mice were analysed it was found that an even larger fraction of the HSV1 DNA was in a nucleosomal form (Deshmane and Fraser 1989). The nucleosomal structure of a 'quiescent' infection in fibroblasts was studied by MNase digestion and analysis of the TK gene (Jamieson *et al.* 1995). No nucleosomal structure was found at this locus.

As this digestion has never been carried out on latently infected cells producing LATs and the nucleosomal structure of the LAT region looked at, it was thought interesting to do so, as well as to further check that the ChIP assay would be relevant to this *in vitro* latent model.

ND7s were infected or mock infected at an MOI of 5 with 1764 4- 27- CMVGFP/5 virus. 27/12/M:4 cells were infected at an MOI = 1. After 3 days for the ND7s and 1 day for the 27/12/M:4 cells, nuclei were isolated using the Nuclei EZ Prep Nuclei Isolation kit (Sigma, Dorset, UK) according to the manufacturer's instructions and digested with 225U micrococcal nuclease (Roche, E. Sussex, UK) for 5, 10 or 15 mins at 37°C. Samples were then treated with proteinase K (10ug/mL) at 37°C for 30 mins and purified by phenol extraction. Samples were then run on a 1.5% agarose gel with equivalent digestions viral DNA and visualised by UV. The gels were then used in Southern blots and probed with a *Not*3.5 fragment of the LAT region (nt 118439 - 122025). Figure 5-10 shows the digested samples prior to blotting and the resulting probed blots.

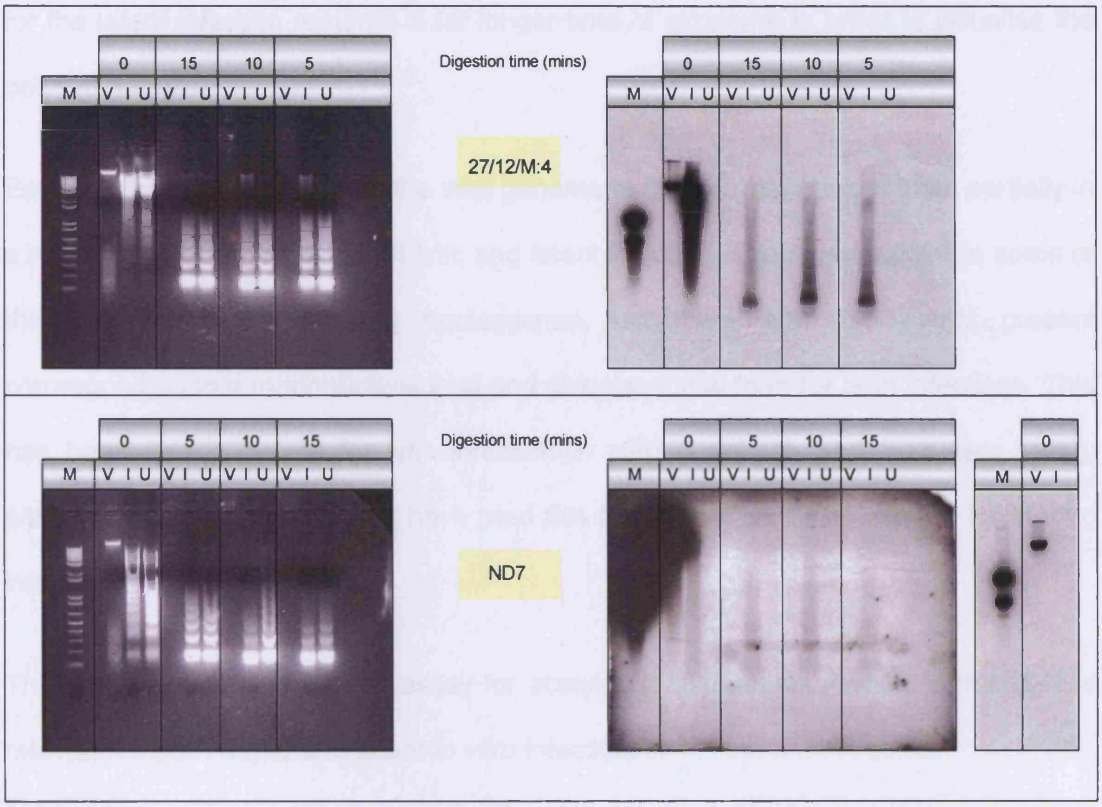


Figure 5-10 Micrococcal nuclease digestion of HSV1 lytic and latent viral chromatin.

ND7 and 27/12/M:4 cells were infected with 1764 4- 27- CMVGFP/5 at an MOI of 5 and 1 respectively and the nuclei harvested at 3 and 1 days post-infection respectively. Nuclei were digested with micrococcal nuclease for the times indicated, along with uninfected cells and viral DNA. The gels were then used for Southern blots, probed with a 3.5kb section of the LAT region. The blot for 27/12/M:4 infected cells was exposed for 1 hour and the blot for ND7 cells exposed for 3 days. The extra section of ND7 blot shown is the first 4 lanes exposed for 1 hour.

The digestion of nuclei with micrococcal nuclease shows the classic nucleosomal ladder pattern in the agarose gels. Digestion for 5, 10 or 15 minutes did not give a great difference in pattern, although overall smaller units of nucleosomes can start to

be seen in the 15 minute digest of the ND7 cells. There are more viral genomes in the lytically infected cells than the latently infected as illustrated by the fact that although the agarose gels for both infections show equivalently bright bands, the Southern blot for the latent infection required a far longer time of exposure in order to visualise the positive samples.

Both Southern blots show that the viral genome at the LAT region is at least partially in a nucleosomal form during both lytic and latent infection. The smears point to some of the viral genome not being nucleosomal, but there are also bands present corresponding to a mononucleosomal and dinucleosomal form for both infections. This has been shown before for lytic infection *in vitro* (Leinbach and Summers 1980), although not by all groups that have tried this (Mouttet *et al.* 1979) but not for *in vitro* latent infection of HSV1.

This result shows that a ChIP assay for acetylated histones associated with HSV1 is relevant for both a lytic and latent *in vitro* infection.

5.4 OPTIMISATION OF THE SONICATION PROCEDURE

5.4.1 Volume of buffer for sonication

The first part of the ChIP protocol that needed optimising was the sonication procedure. The volume in which to sonicate the crosslinked samples needed to be looked at. The protocol stated that 200μL of lysis buffer should be used, but using this volume resulted in frothing of the sample, even after turning the power of the sonicator down, and further sonication was not possible. To increase the volume of sample, instead of adding more lysis buffer, which would have altered the final SDS concentration, ChIP dilution buffer was used. As the subsequent step to sonication

was dilution of the sample with this buffer anyway, it seemed reasonable to add this prior to sonication and adjust the volume of the buffer added afterwards. In order to keep the volume to a minimum, a range of volumes from 300 - 1000μL, in 100μL increments was tested. This was carried out on samples of non-infected 27/12/M:4 cells that had been cross-linked as per the first part of the protocol.

Volumes below 600μL had a tendency to froth, whereas those above did not and sonication was possible. Therefore, 400μL of dilution buffer was chosen as the volume of ChIP dilution buffer to add to the sample prior to sonication, and then afterwards, the remainder of the 1800μL total volume of buffer (i.e. 1400μL) would be added.

5.4.2 Time of sonication

The aim of the sonication step is to disrupt the chromatin into smaller fragments that may be immunoprecipitated. There seems to be varying opinion on the desired size of chromatin post-sonication, ranging from <1.5kb to 300bp. Supposedly the smaller they are, the better the resolution of the assay. Using a probe sonicator on 30% power for 40-second bursts; sonication of infected cells was attempted. Wells of 1 x 10^6 27/12/M:4 cells were infected at an MOI of 1 with 1764 4- 27- pR19GFP virus and ND7 cells were infected at an MOI of 5 with 1764 4- 27- CMVGFP/5 virus and a range of the number of sonication bursts was carried out, resting the samples on ice between each one to keep the samples cool. The sonication procedure was also carried in a cold room to minimise heating of the samples.

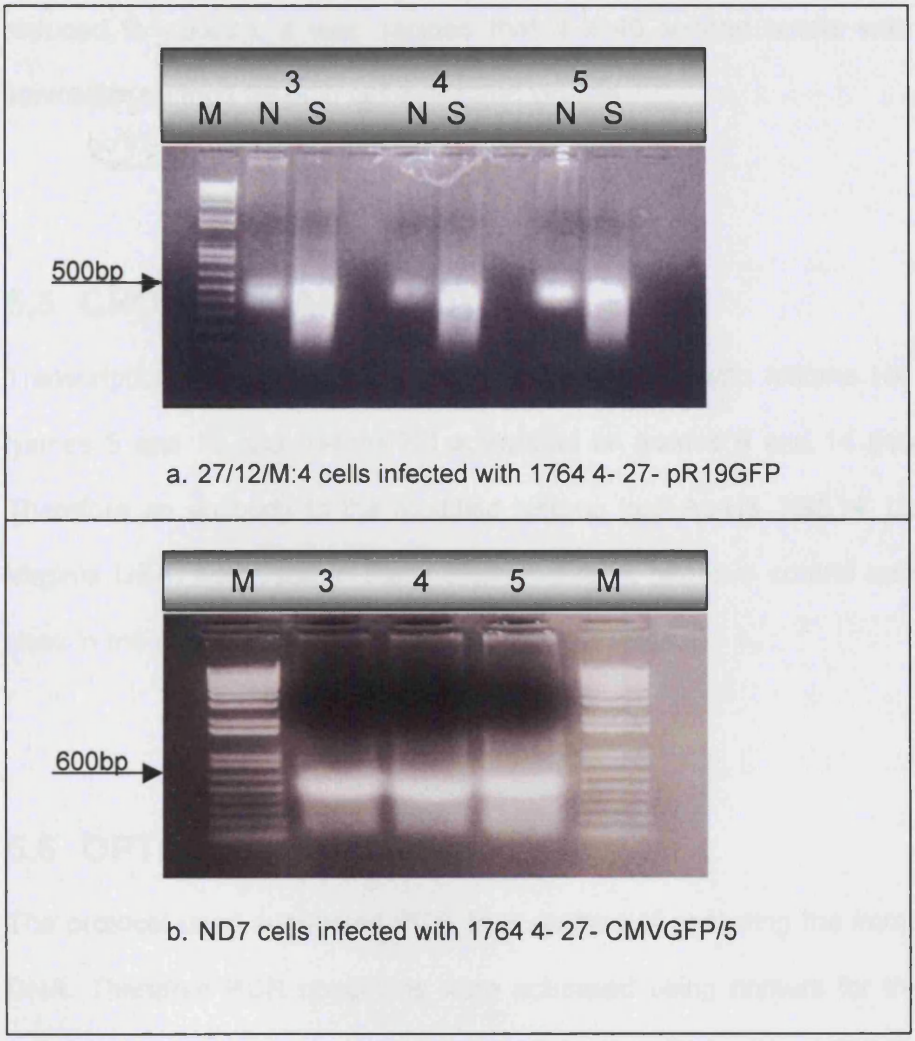


Figure 5-11 Sonication of infected samples

Samples were sonicated for bursts of 40 seconds, the number above indicating for how many. M = 1 kb+ DNA marker. Panel a. N = non-sonicated, S = sonicated.

Figure 5-10 shows that it did not appear to make a great difference if 3, 4 or 5 bursts of 40 seconds were applied to the samples. The difference in average size of chromatin pre-and post-sonication can be seen in fig 5-11a, although it was expected that the pre-sonication band would be larger than it was. As all sonicated fragments were

reduced to <600bp, it was decided that 4 x 40 second bursts was a reasonable sonication method for both cell types and viruses.

5.5 CHOICE OF ANTIBODY

Transcriptionally permissive chromatin is associated with histone H4 acetylated on lysines 8 and 16 and histone H3 acetylated on lysines 9 and 14 (Kuo *et al.* 1996). Therefore an antibody to the modified histone (anti-Ac-H3, K9&14, Upstate Biotech, Virginia USA) was used in these experiments. A negative control antibody was also used in the optimised assays– chicken anti-IgG (Sigma).

5.6 OPTIMISATION OF PCR

The protocol used suggested PCR as a method of analysing the immunoprecipitated DNA. Therefore PCR conditions were optimised using primers for the promoters of interest. These were chosen as LAP1, LATP2-5', LATP2-mid, LATP2-3', the promoters for the IE protein ICP0, the early gene for TK and CMV. US5, where the CMVGFP cassette is inserted is an early gene and it may be expected that any promoter inserted would take on the expression timings of an early gene. TK is also an early gene and therefore the promoter should be transcriptionally active at a similar time to the US5 gene. Thus acetylation of the CMV promoter in the 1764 4- 27- CMVGFP virus can be compared to that of the TK promoter. Viral DNA 1764 4- 27- pR19GFP was used in the optimisation. Conditions for the cellular gene promoters Adenine phosphoribosyl transferase (APRT) and cyclophilin were also optimised using genomic DNA from ND7 cells.

The target sequences had to be relatively small, as the sonicated fragments were ~600bp in general. Where possible primers were designed to amplify a sequence of no more than 200bp.

Table 5-2 shows the primers whilst figure 5-12 shows the resulting PCR bands and conditions used.

PROMOTER REGION	FORWARD PRIMER	REVERSE PRIMER
LAP1	TGCCCGCGAGATATCAAT	CACGTGGGTAGGTGATGTAA
LATP2-5'	TCGGCGACATCCTCCCCCTAA	GAAACATTCCGGCGACGGAA
LATP2-MID	TGAGATGAACACTCGGGGTTAC	TGGTGTGCTGTAACACGAG
LATP2-3'	CCCTAAAGTTGTTCCTAAAGC	AGGGGATGGGCGTGTTGGTTA
ICP0	ATTGGGGGAATCGTCACTG	AGGTGCAAATGCGACCAGA
TK	CTATGATGACACAAACCCCG	CCACACGCGTCACCTTAATA
CMV	ATCATATGCCAAGTACGCCC	GTGCCAAAACAAACTCCCAT
APRT	GGCAAGCGTACAAAATGCAA	TGAGGTGGTGGAGTGTGTGA
CYCLOPHILIN	CGAGCTGTTTGCAGACAAAG	TTCTTGCTGGTCTTGCCATT

Table 5-2 PCR primers used for detection in the ChIP assay

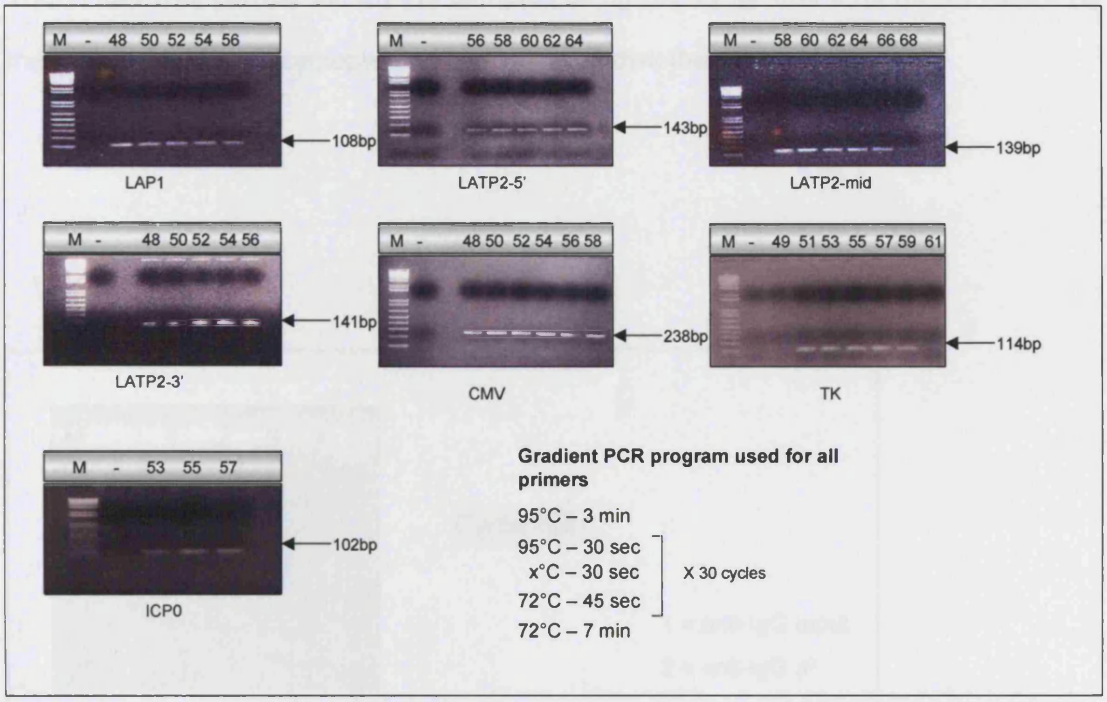


Figure 5-12 Optimisation of PCR conditions for primers used in ChIP assays

1764 4- 27- pR19GFP viral DNA was used as the template for all of the reactions. Amplified DNA size is indicated for each set of primers.

All primer sets worked at most annealing temperatures tested. Temperatures used subsequently were picked from the middle of the tested range, except where stronger bands were seen for one temperature of the range as for the LAP1 primers.

5.7 VALIDATION OF THE ASSAY

To validate the assay uninfected cells were used in a full ChIP assay with primers for cellular constitutively active genes. 3 x 3.5cm^2 wells of ND7 cells were used in the assay – crosslinked, sonicated, immunoprecipitated, cross-links reversed and cleaned. Then PCR was carried out on the samples using the conditions determined above for the genes APRT and cyclophilin. Figure 5-13 shows the result of the assay.

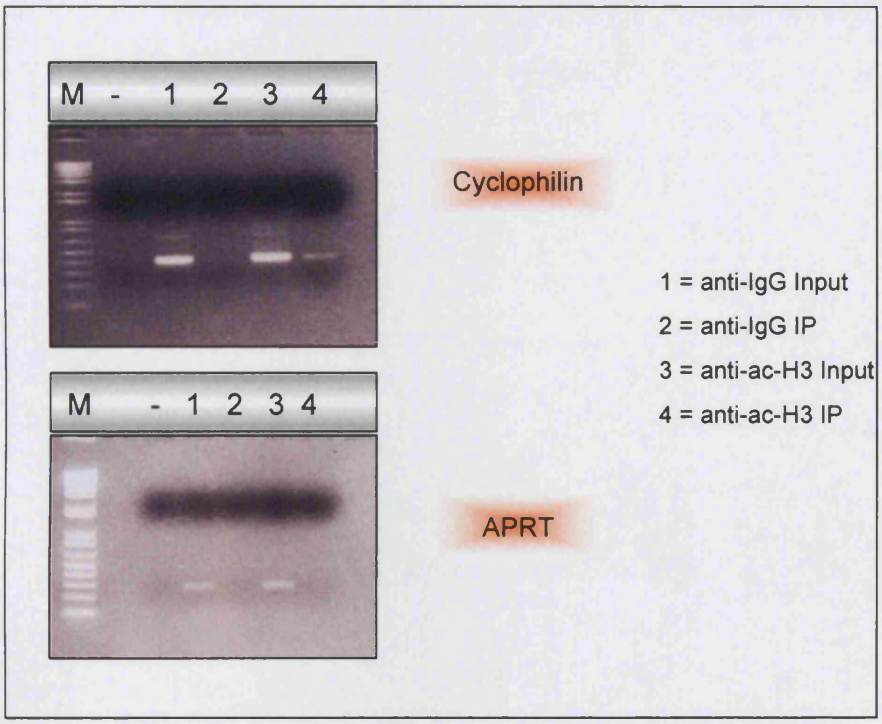


Figure 5-13 PCR for ChIP assay validation

PCR was carried out using the program described in fig. 5-12 above for 30 cycles with primers under optimised conditions. 10μL of each sample was run on a 1.5% agarose gel. M = 1kb+ DNA Marker, - = PCR negative control.

The results show that the assay has worked. The input samples contain the genes and whilst the APRT gene has not been precipitated, the gene for cyclophilin has (top, lane 4), showing that it is associated with acetylated histone H3 and therefore transcriptionally active in these cells. It is unexpected that APRT does not appear to be positive for acetylation of this histone, but as the reason for this is not relevant in this study it was pursued no further.

5.8 INITIAL CHIP ASSAYS

5.8.1 Analysis of samples by PCR

Firstly the detection of the viral chromatin in the input samples (i.e. after sonication, before precipitation) was obviously required if it was to be detected downstream in the assay after precipitation. To check this 3 x 3.5cm^2 wells per sample of 27/12/M:4 and ND7 cells were infected with 1764 4- 27- CMVGFP-5 virus at an MOI of 1 and 5 respectively. The first steps of the ChIP assay were carried out up to the point of sonication. The samples then had the cross-links reversed and were cleaned up. They were then used in a PCR reaction with the CMV primers, as optimised above, at 54°C annealing temperature. Figure 5-14 shows the results.

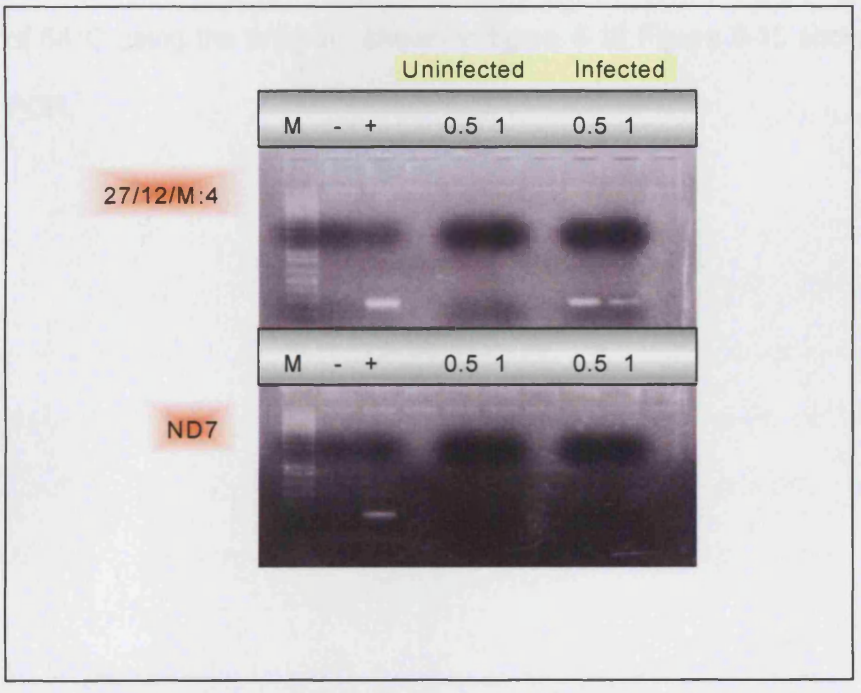


Figure 5-14 Agarose gel from PCR with CMV primers of input samples from uninfected and infected cell lines.
0.5 and 1 μL of each sample was used per PCR reaction as indicated. 10μL of each PCR reaction run on 1.5% agarose gel. M = 1kb+ DNA ladder, - and + = negative and positive viral DNA PCR controls (1764 and 1764/CMV/GFP respectively).

The PCR of the input samples show that the lytic virus sample is picked up by PCR but the latent virus is not. It was apparent that either a higher MOI of virus would need to be used for latent infection, or a more sensitive detection method used. As using more virus was not desirable due to the large amounts required for the assays and also the health of the cells being infected, it was decided that a more sensitive detection method would be attempted.

It was decided to see if any of the lytic virus when precipitated would be detectable by PCR, so the same infections were carried out on both 27/12/M:4 and ND7 cells as before. This time the full ChIP assay was carried out and the samples cleaned up. Higher volumes of input sample were used in the PCRs than before – 1 and 5μL as opposed to 0.5 and 1μL in the hope of detecting the latent samples. The precipitated samples were also used at 1, 5 and 10μL in the PCRs to increase chances of observing positive samples. Primers for the CMV promoter were again used with a T_m of 54°C using the program shown in figure 5-12 Figure 5-15 shows the results of the PCR.

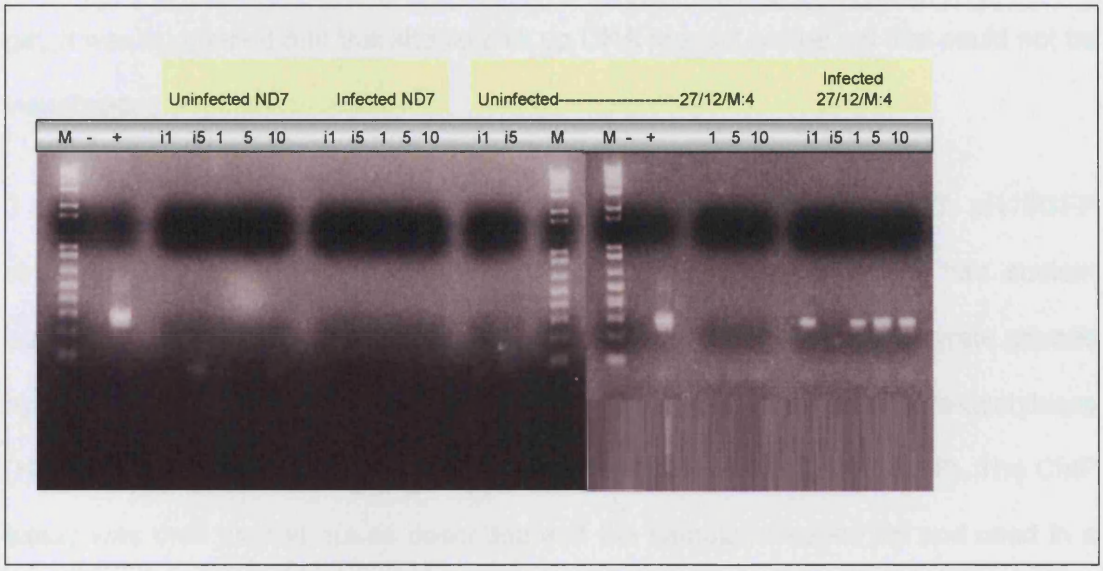


Figure 5-15 PCR with CMV primers from ChIP assay of 1764 4- 27- CMVGFP-5 virus in lytic and latent infections.

10μL of each PCR was run on a 1.5% agarose gel. i1 and i5 = 1 and 5 μL of input sample used in the PCR respectively. 1, 5 and 10 are the volumes of IP samples used in the PCR. – and + are viral DNA PCR controls.

As shown in figure 5-15 the immunoprecipitated (IP) samples are positive for the CMV promoter in 27/12/M:4 cells, suggesting that it is acetylated during the lytic infection. Again the input samples for the latent infection did not show up by PCR. Although the result for the lytic infection was encouraging there was no point in analysing the samples further until the problem of detection in the latent samples had been resolved.

5.8.2 Analysis of samples by PCR and Southern blot

To improve detection of the latent samples in ND7 cells without having to use more virus per infection, it was decided to try PCR followed by a Southern blot of the PCR gel. It was suggested that this should pick up DNA present on the gel that could not be visualised.

3 x 3.5cm^2 wells of ND7 cells were infected at an MOI of 5 with 1764 4- 27- pR19GFP virus and left for 3 days. 2 days post-infection, one set of infections had sodium butyrate added to the media at a final concentration of 3mM. Sodium butyrate causes hyperacetylation of histones (Riggs *et al.* 1977) by inhibition of histone de-acetylases (HDACs) (Boffa *et al.* 1978; Candido *et al.* 1978; Sealy and Chalkley 1978). The ChIP assay was then carried out as described and the samples cleaned up and used in a PCR firstly for the CMV promoter and also for the TK promoter using the program shown in figure 5-12. As previously, no positive bands were seen for any of the input or IP samples (see figure 5-16). The gels were then used for a Southern blot (see section 2.2.8) each with a probe for the relevant promoter. Probes were made from the PCR product with the relevant primers using 1764 4- 27- pR19GFP viral DNA as a template and labelled as in 2.2.3.8.2. The gels and blots are shown in figure 5-16.

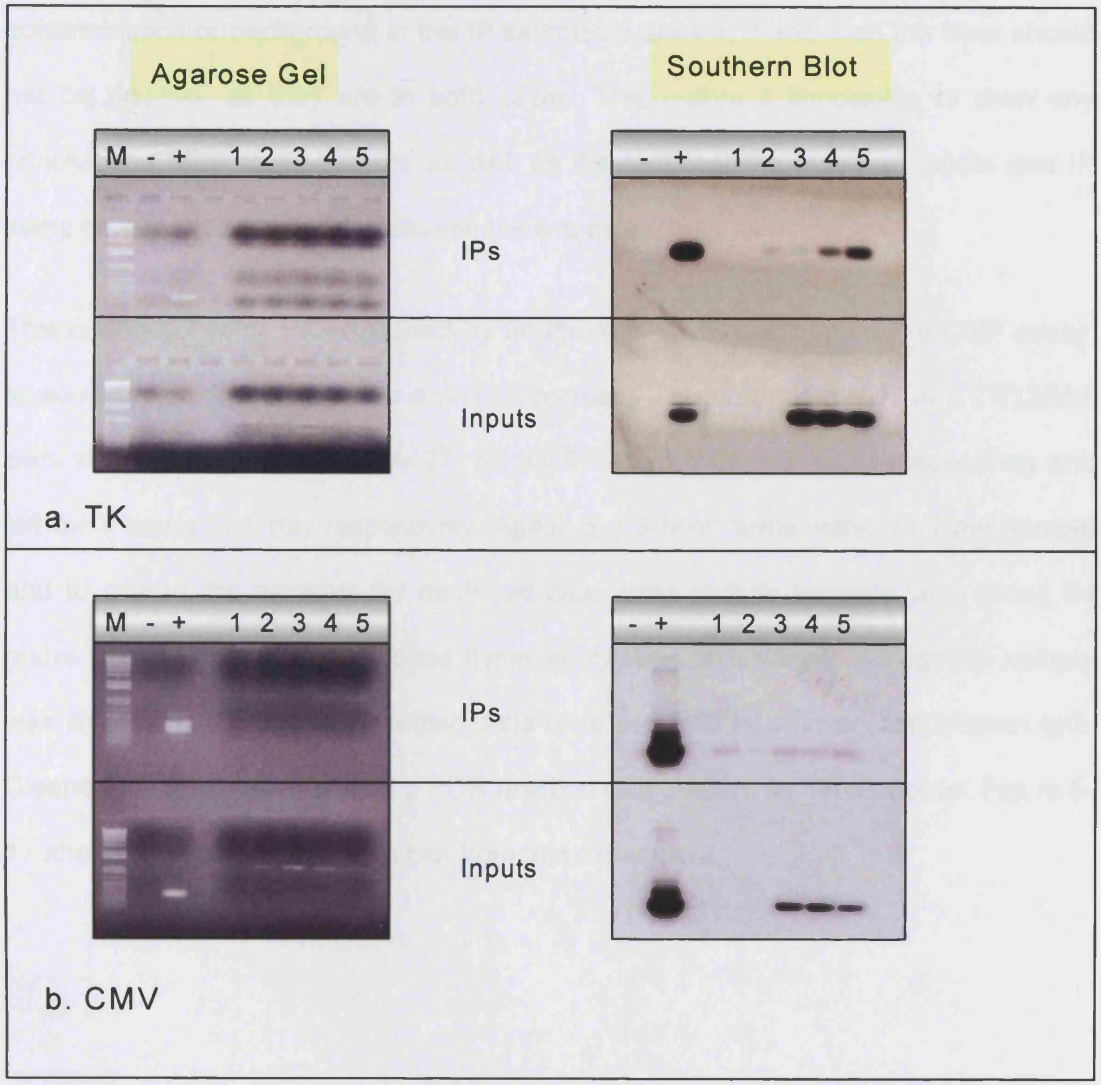


Figure 5-16 ChIP assay on 1764 4- 27- pR19GFP in ND7 cells 3 days p.i.

Agarose gels for PCR and corresponding Southern blots for the same samples for a. TK and b. CMV. 10μL of each PCR reaction was run on a 1.5% agarose gel and then used in a Southern blot with an appropriate probe for each promoter of interest.
1 = ND7 cells only (no antibody), 2 = ND7 cells + anti-acH3, 3 = Infected cells (no antibody), 4 = Infected cells + anti-acH3, 5 = infected cells + sodium butyrate + anti-acH3.
(NB The bands in the gel in panel b. are non-specific and do not come up on the southern blot.)

Using the PCR gel for a Southern blot does therefore appear to give a more sensitive method of detection. The bands on the blots are positive and yet cannot be seen in the PCR gel. However, there is a problem in that because it is so sensitive there is some

contamination or background in the IP samples. Lanes 1, 2 and 3 on the blots should not be positive, as they are in both cases. This makes it impossible to draw any conclusions from these results, as well as the fact that the levels of inputs and IP samples are not comparable between the two gels.

This method of using PCR followed by Southern blot was used again in a ChIP assay, to see if the contamination was a one-off problem. This time, both ND7 and 27/12/M:4 cells were infected with 1764 4- 27- pR19GFP at an MOI of 5 and 1 respectively and left for 3 day and 1 day respectively. Again, 3 x 3.5cm^2 wells were used per sample and to one of the samples for each cell type 3mM sodium butyrate was added 24 hours prior to the assay to induce hyper-acetylation of histones. A negative sample was also included in which infected cells were precipitated with an anti-chicken IgG. Cleaned samples were used in a PCR reaction with primers for TK as above. Figure 5-17 shows the gel and Southern blot from this experiment.

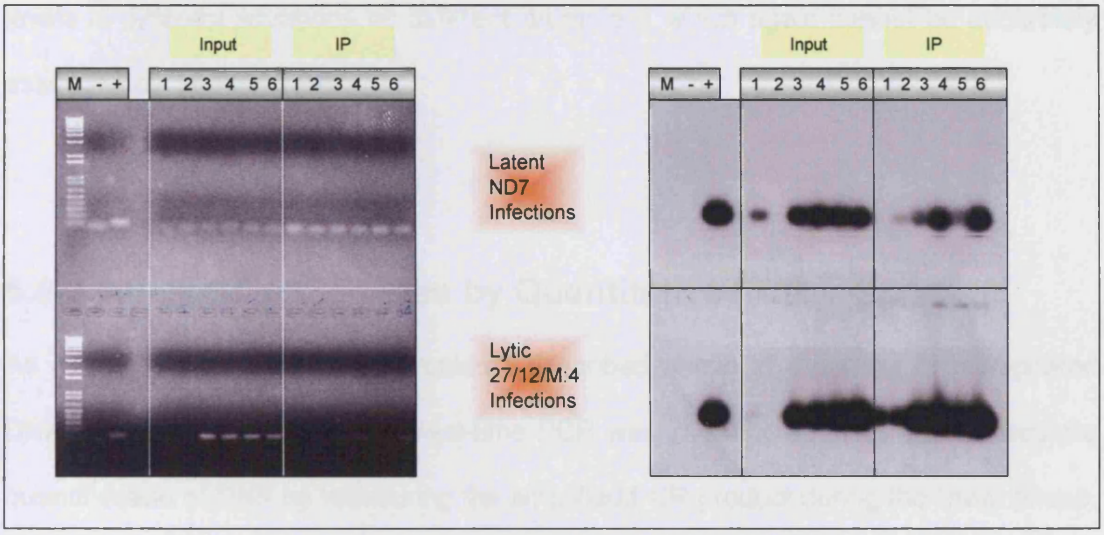


Figure 5-17 ChIP assay on 1764 4- 27- pR19GFP on ND7 and 27/12/M:4 cells.

Agarose gel for PCR and corresponding Southern blot. 10μL of each PCR reaction was run on a 1.5% agarose gel and then used in a Southern blot with an appropriate probe for the TK promoter.
1 = Cells only (no antibody), 2 = Cells + anti-acH3, 3 = Infected cells (no antibody), 4 = Infected cells + anti-acH3, 5 = infected cells + anti-IgG, 6 = infected cells + sodium butyrate + anti-acH3.

Figure 5-17 shows that the lytically infected samples do not require the Southern blot to detect the IPs as the results can be seen from the agarose gel alone. This also highlights the contamination or background problem with the Southern blots, as when the corresponding blot is looked at there are bands in the 'negative' samples. Again this is seen in the latently infected ND7 samples and although the contaminating bands are probably just background, it is not ideal as it makes it difficult to judge a positive result.

The other two drawbacks of this method of analysis are that firstly there is no way of quantitatively comparing results between different promoters and different infections. Secondly, the acetylation is probably not just 'on' or 'off' such that there will be differing levels in different situations on different promoters, which again cannot be accurately assessed by Southern blot.

5.8.3 Analysis of Samples by Quantitative PCR

As a possible solution to the problems described above in detection of precipitated DNA, the use of quantitative or real-time PCR was investigated. This allows accurate quantification of DNA by measuring the amplified PCR product during the linear phase. By running the samples with multiple primer sets simultaneously should also allow a direct comparison of the acetylation profile of different promoters in the same sample.

5.8.3.1 Primer Validation

As the primers used above to optimise the standard PCR had different melting temperatures (section 5.6) and the quantitative PCR (qPCR) cycle normally runs at 60°C annealing temperature, firstly all of the viral primers were used in a PCR reaction on 1764 4- 27- CMVGFP/5 viral DNA to ensure that they could still be used. The program shown in figure 5-12 was used as before. Figure 5-18 shows the PCR products on a gel.

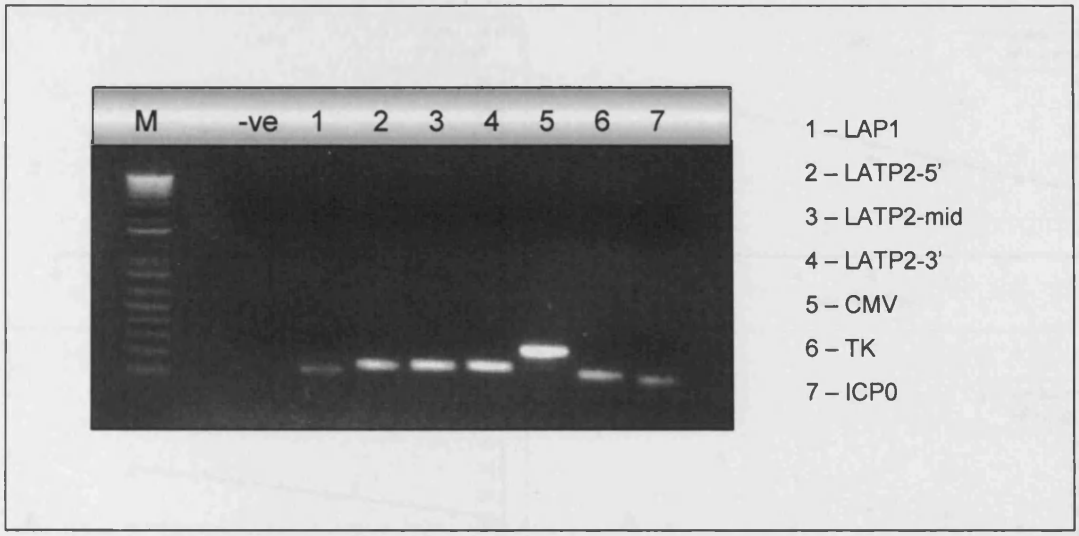


Figure 5-18 PCR of 1764 4- 27- CMVGFP/5 viral DNA using all primers at 60°C annealing temperature.

10μL of each sample was run on a 1.5% agarose gel.

Figure 5-18 shows that all the primers should be able to be used at 60°C. Any variations in PCR efficiency between primer sets will not matter in the qPCR as the difference between input samples and IP samples will be measured for each primer set used.

The same viral DNA and primers were then used in a qPCR reaction on an ABI7000 real-time PCR machine as described in section 5.2.3. The same 1764 4- 27- CMVGFP/5 viral DNA was used for the standard curves with serial dilutions from 1 – 1 x 10^{-5} μL. Figure 5-18 shows the standard curves generated by plotting the log volume of viral DNA against the Threshold Cycle (C_T).

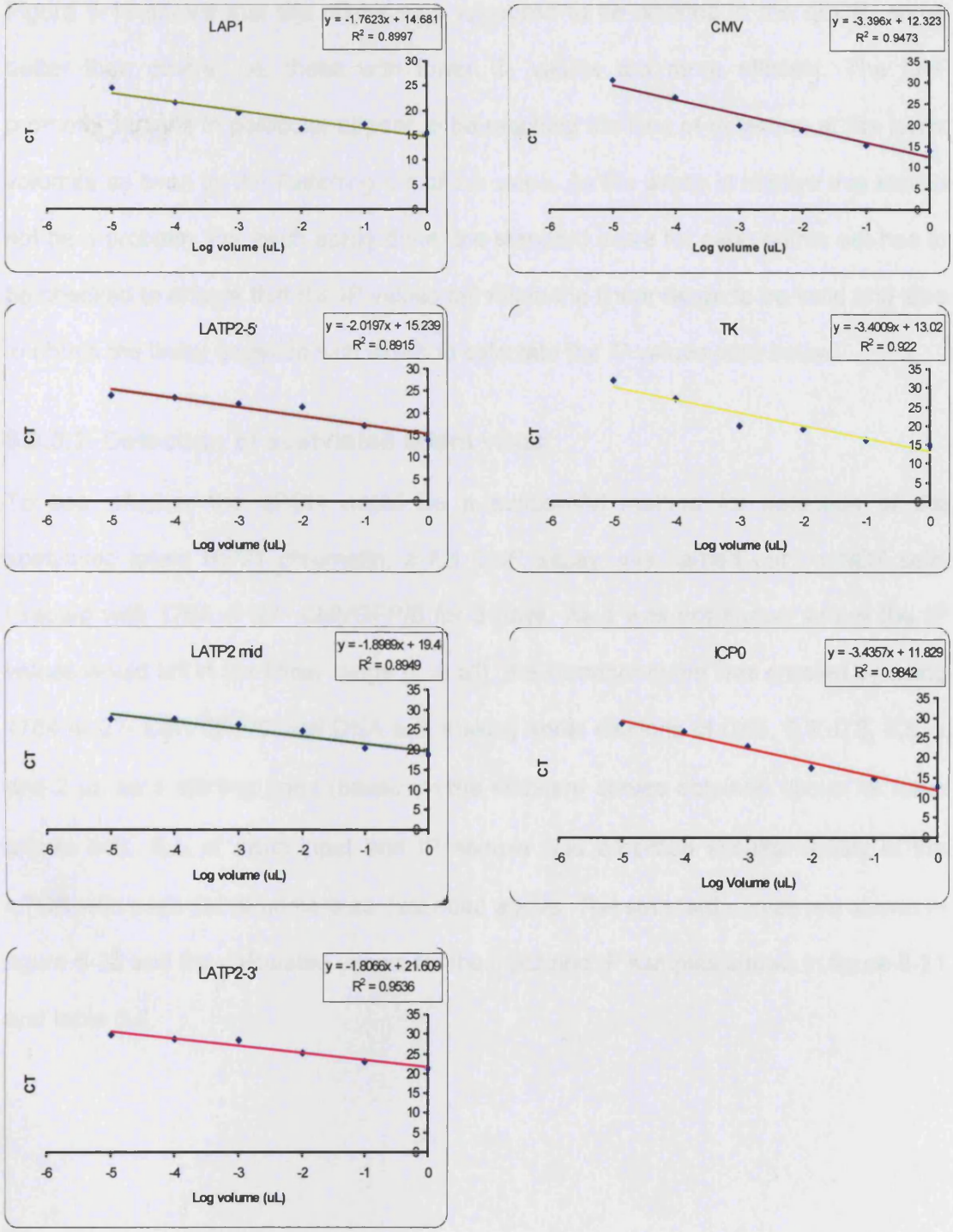


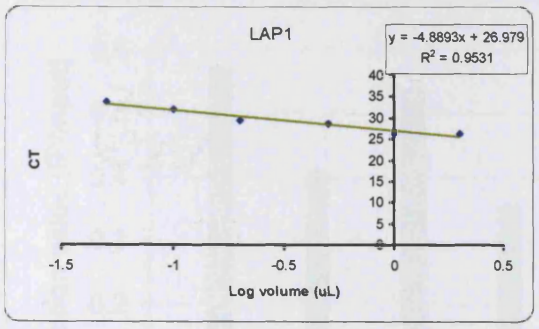
Figure 5-19 Standard curves generated by qPCR

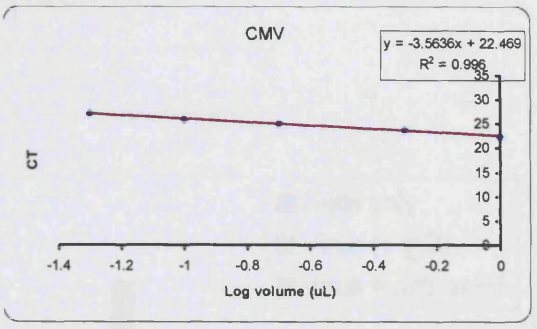
qPCR was carried out on viral DNA with primers for the different promoters of interest simultaneously. The linear equation given by each primer set and the R^2 value is shown on each chart.

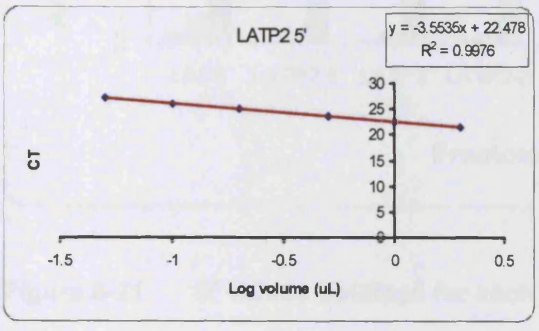
Figure 5-19 shows that the primer sets appeared to be working in the qPCR, some better than others, i.e. those with lower C_T values are more efficient. The LAT promoter regions in particular appear to be reaching the limit of detection at the lower volumes as seen by the flattening out of the slope. As the assay is relative this should not be a problem. For each assay done, the standard curve for each primer set has to be checked to ensure that the IP values fall within the linear range to be valid and also to obtain the linear equation with which to calculate the IP values (see below).

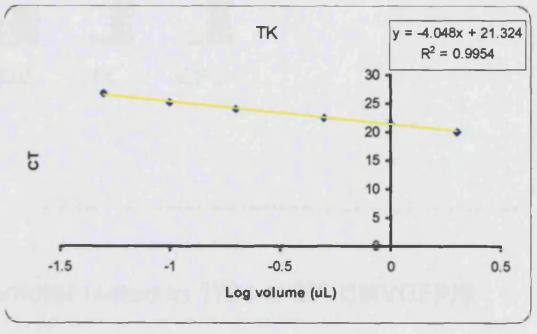
5.8.3.2 Detection of acetylated latent virus

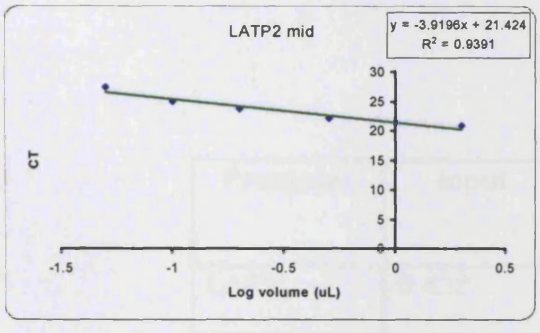
To see whether the qPCR would be a successful method for detection of the acetylated latent HSV1 chromatin, a full ChIP assay was carried out on ND7 cells infected with 1764 4- 27- CMVGFP/5 for 3 days. As it was not known where the IP values would fall in the linear range (if at all), the standard curve was created by using 1764 4- 27- CMVGFP/5 viral DNA and making serial dilutions of 0.05, 0.1, 0.2, 0.5, 1 and 2 μL as a starting point (based on the standard curves obtained above for each primer set). 1μL of each input and IP sample was amplified simultaneously in the qPCR with each set of primers as described above. The standard curves are shown in figure 5-20 and the calculated values for the input and IP samples shown in figure 5-21 and table 5-3.

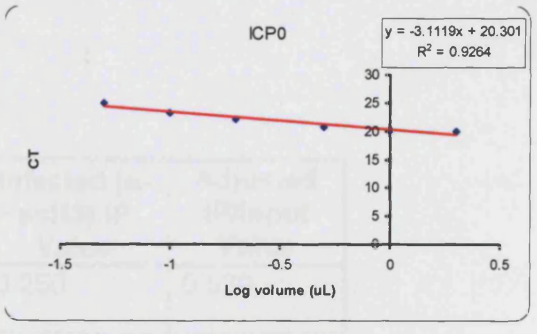












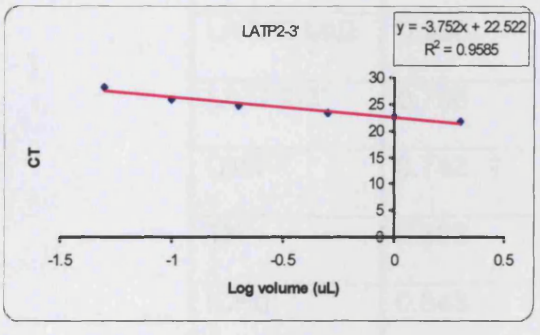


Figure 5-20 **Standard curves for ChIP assay on ND7 cells infected with 1764 4- 27-CMVGFP/5 3 days p.i.**

NB: The highest viral DNA concentration sample for the CMV primer set was lost, therefore the graph only has 5 points as opposed to 6.

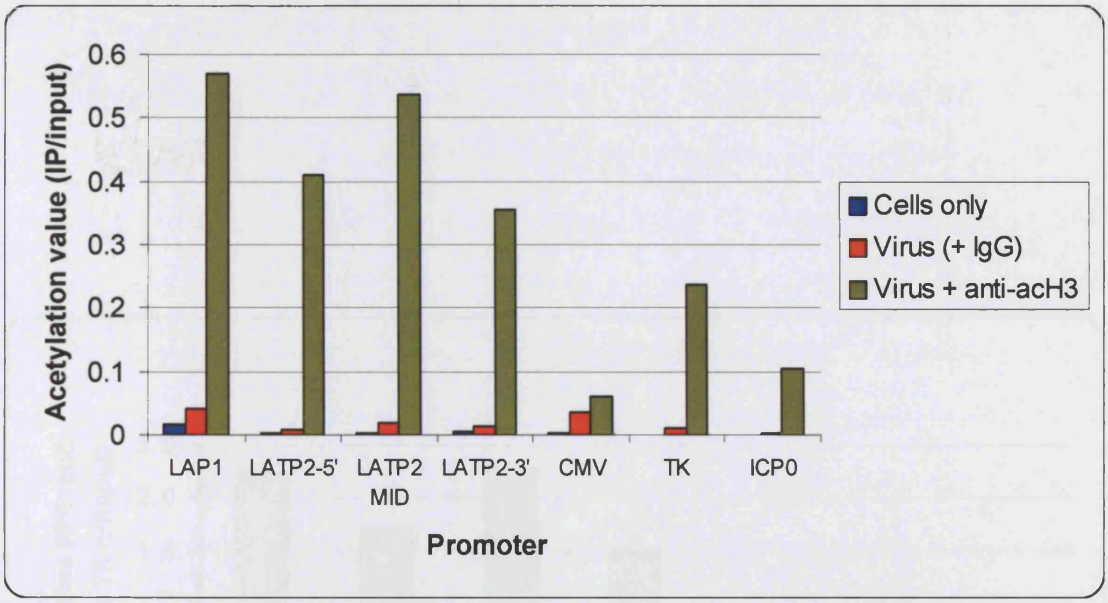


Figure 5-21 **IP values obtained for each promoter tested in 1764 4- 27- CMVGFP/5**

Promoter	Input	Infected (α-acH3) IP Value	Adjusted IP/Input Value
LAP1	0.438	0.250	0.529
LATP2-5'	0.381	0.156	0.409
LATP2 MID	0.968	0.521	0.538
LATP2-3'	0.796	0.355	0.355
CMV	0.742	0.045	0.024
TK	0.698	0.165	0.236
ICP0	0.843	0.088	0.104

Table 5-3 **IP values from qPCR for ND7 cells infected with 1764 4- 27- CMVGFP/5 3 days p.i.**

IP values are calculated using the formula y= mx + c with the values for the standard curve for each set of primers used shown on the charts in figure 5-20. The adjusted values account for any background given by the samples precipitated with the control α-IgG antibody falling within the linear range (only LAP1 and CMV in this case)

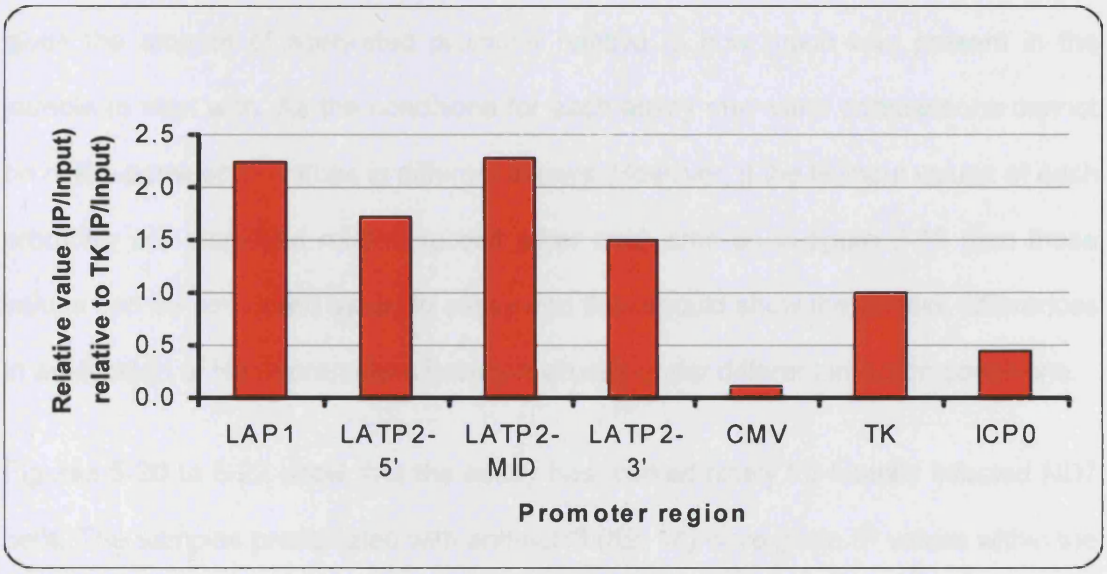


Figure 5-22 **Relative acetylation of histone H3 at promoters in 1764 4- 27- CMVGFP/5 3 days p.i. in ND7 cells compared to at the TK promoter.**

The relative acetylation is calculated by dividing the IP value for any given promoter by that of the TK promoter.

The standard curve for each primer set in fig 5-20 gives a linear equation that is used to calculate the IP values of the samples from their C_T values. As x is the log volume of the viral DNA standard and y is the C_T, the IP value can be calculated as:

$$\text{Log IP} = \frac{(C_T - c)}{m}$$

The value is relative to the volume of viral DNA used for the standard curve, but as the same viral DNA was used for all of the primer sets, they can be compared to each other. The input value is the amount of the promoter in the sample prior to immunoprecipitation and the IP value is the amount of promoter associated with acetylated histone H3 in the sample after immunoprecipitation, thus the ratio IP/input gives the amount of acetylated promoter relative to how much was present in the sample to start with. As the conditions for each assay may vary, comparisons cannot be made between IP values in different assays. However, if the IP/input values of each promoter are displayed relative to one other each time as in figure 5-19 then these values can be compared assay to assay and thus should show the relative differences in acetylation of H3 at promoters between viruses under different infection conditions.

Figures 5-20 to 5-22 show that the assay has worked nicely for latently infected ND7 cells. The samples precipitated with anti-acH3 (K9, 14) have given IP values within the linear range of the standard curves and the negative control samples are out of range and low. This was exciting as at the time it was the first evidence that HSV1 chromatin can be acetylated and that there are potential differences between lytic and latent regions.

5.8.3.3 Detection of acetylated lytic virus

As the detection was successful for the latent virus a ChIP assay was also carried out on lytically infected cells to check that qPCR can be used to detect acetylated promoters. It was likely that they would be as there is generally more viral chromatin present in the lytic samples than the latent as seen in section 5.3.4.

The standard curve was created by using 1764 4- 27- pR19GFP viral DNA and making serial dilutions to 10^{-1} to 10^{-6}μL as it was known that the viral DNA was more concentrated than the viral DNA used in the latent assay standard curve (from visualisation on an agarose gel). 1μL of each input and IP sample was amplified

simultaneously in the qPCR with each set of primers as described above. The standard curves are shown in figure 5-23 and the calculated values for the input and IP samples shown in figure 5-24 and table 5-4.

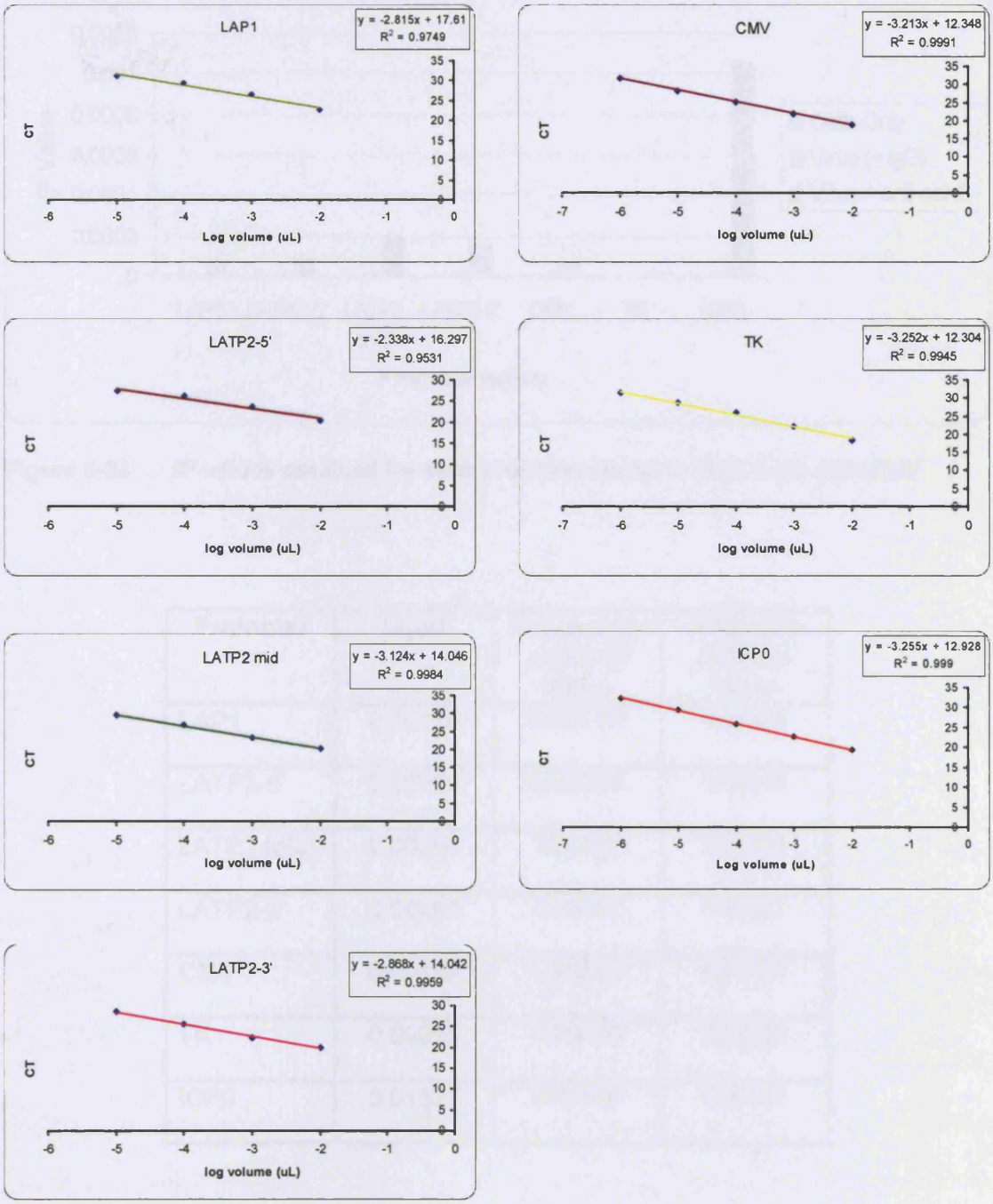


Figure 5-23 Standard curves for ChIP assay on 27/12/M:4 cells infected with 1764 4-27- pR19GFP 1 day p.i.

The standard curves for the LAT promoter regions only have 4 points as opposed to the other standard curves. This is because for all four primer sets, the lowest dilution of viral DNA had a C_T value equal or below the negative control C_T and was therefore excluded from the graph.

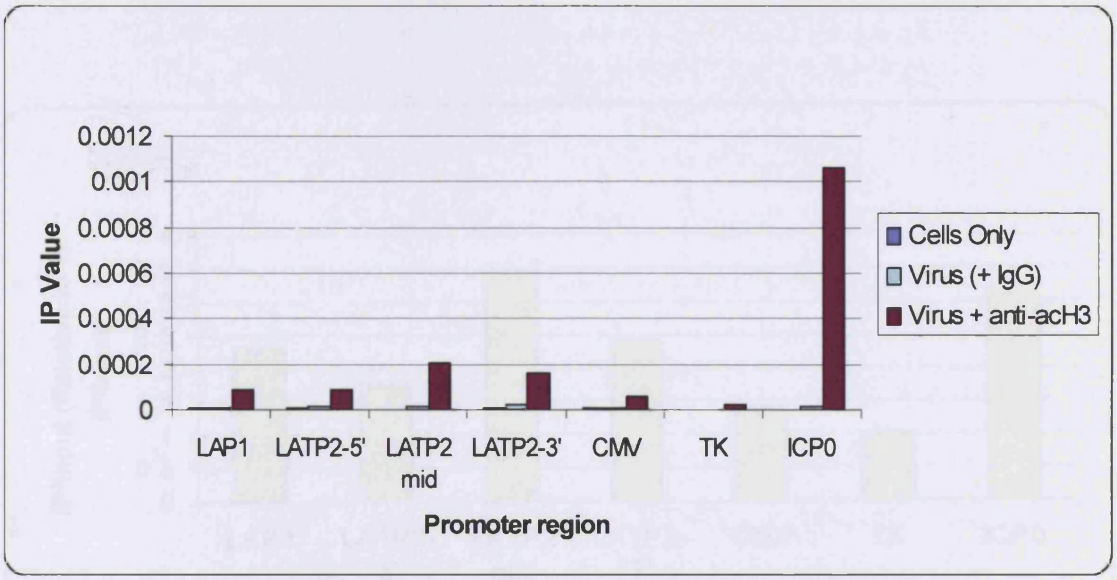


Figure 5-24 **IP values obtained for each promoter tested in 1764 4- 27- pR19GFP**

Promoter	Input	Infected (α-acH3) IP Value	Adjusted IP/Input Value
LAP1	0.00175	8.92E-05	0.05092
LATP2-5'	0.00229	8.96E-05	0.03916
LATP2 MID	0.00266	0.00021	0.07806
LATP2-3'	0.00299	0.00016	0.05337
CMV	0.00188	5.97E-05	0.03184
TK	0.00095	2.11E-05	0.02232
ICP0	0.01527	0.00106	0.06947

Table 5-4 **IP values from qPCR of 27/12/M:4 cells infected with 1764 4- 27- pR19GFP 1 day p.i.**

IP values are calculated using the formula y= mx + c with the values for the standard curve for each set of primers used shown on the charts in figure 5-20. The adjusted values account for any background given by the samples precipitated with the control α-IgG antibody falling within the linear range (none in this case).

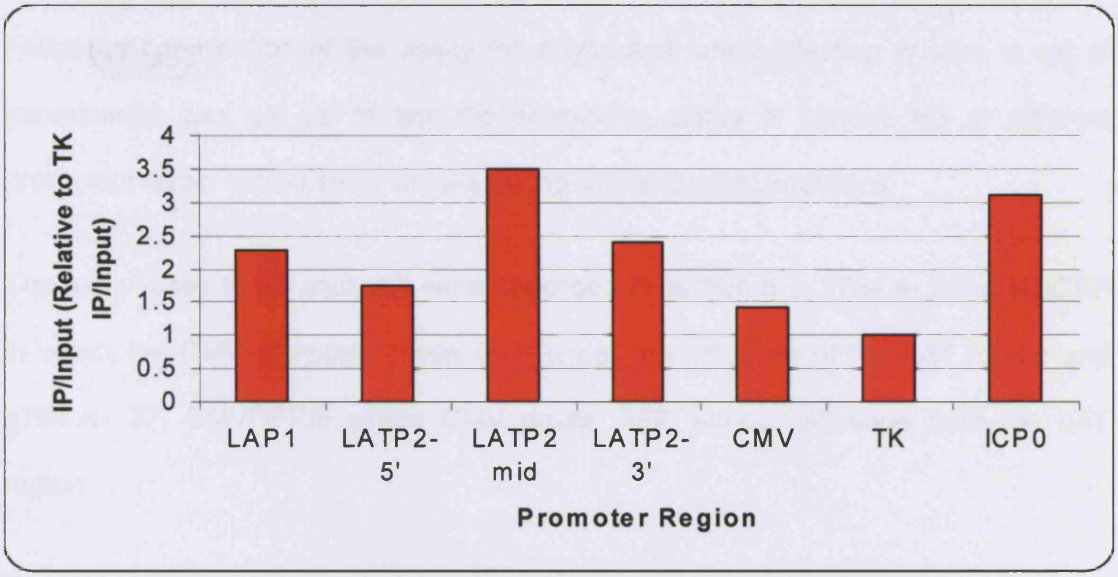


Figure 5-25 Relative acetylation of histone H3 at promoters in 1764 4- 27- pR19GFP 1 day p.i. in 27/12/M:4 cells compared to at the TK promoter.

The relative acetylation is calculated by dividing the IP value for any given promoter by that of the TK promoter.

The figures above show that the assay is also usable for the lytic virus infection. From the IP/input ratios values given in tables 5-3 and 5-4 for the two assays, there appears to be a smaller proportion of the total promoter acetylated in the lytic infection than the latent for all promoters studied.

These were only preliminary experiments and were done in singular and therefore are not suitable for drawing conclusions, however there are possible differences in acetylation between the LAT and non-LAT promoters during latent infection, which prompted further investigation.

5.9 USE OF THE OPTIMISED CHIP ASSAY TO ANALYSE THE ACETYLATION OF TWO HSV1 VECTORS GENOMES

Following optimisation of the assay for a lytic and latent infection *in vitro*, a set of experiments was set up to test the acetylation status of histone H3 at different promoters within two different viruses during lytic and latent infections.

The two viruses to be analysed were described in section 5.1: 1764 4- 27- pR19GFP in which the CMV promoter drives GFP under the influence of the LAT region and 1764 4- 27- CMVGFP/5 where CMV drives GFP without influence from the LAT region.

To study acetylation during acute infection, an earlier time of 3 hours was examined rather than 1 day, after work was published in which the promoters for ICP0, TK and VP16 were shown to all be associated with acetylated histone H3 at 3 hours p.i. but that by 6 hours p.i. only the ICP0 promoter was still acetylated in an *in vitro* lytic model (Kent *et al.* 2004). The same report showed by micrococcal nuclease digestion that by 3 hours p.i. *in vitro*, viral DNA is assembled in a chromatin-like structure.

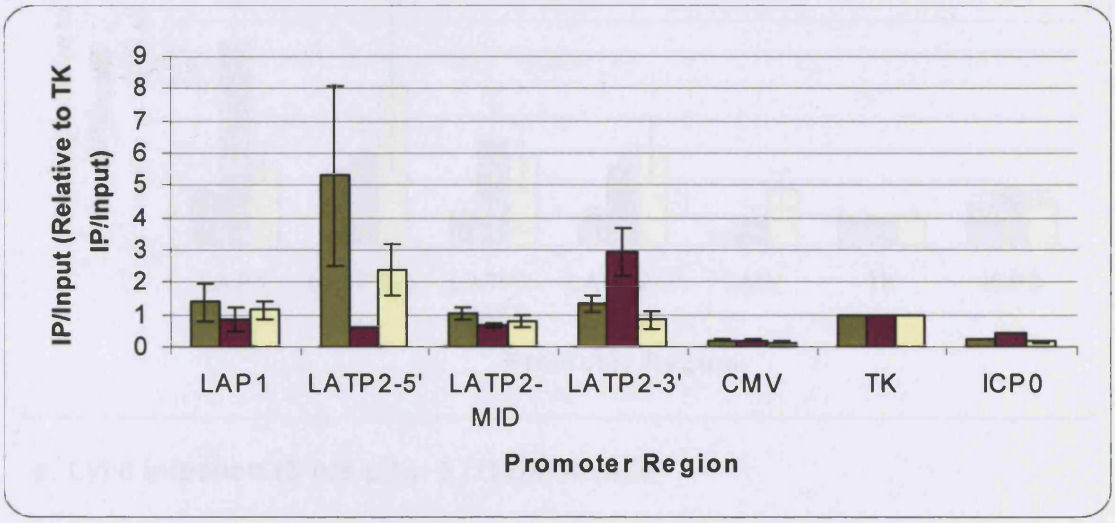
To study acetylation of the viral genomes during latent infection, infected cells were processed after 3 days as before.

The assays were carried out as described above, in both 27/12/M:4 cells and ND7 cells for each virus. The immunoprecipitated samples and inputs from each assay were then used in qPCR reactions with the primers for promoters as described in section 5.6.

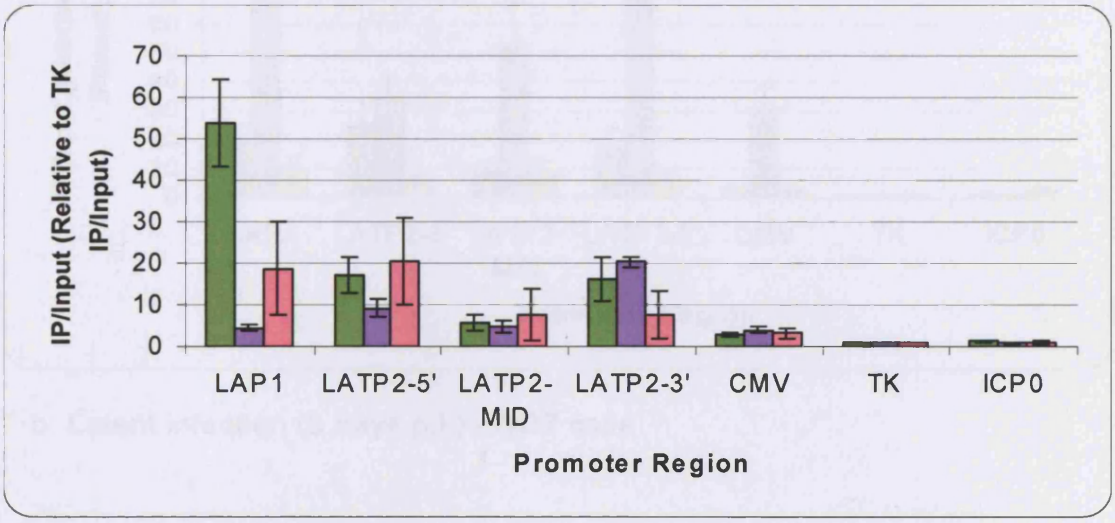
Each assay was carried out in triplicate and for each repeat the samples were used in a qPCR 3 separate times.

This generated 9 sets of ChIP data for each virus in both lytic and latent infections. The averaged relative acetylation values from the three qPCR runs for both lytic and latent assays are displayed in figure 5-26 for 1764 4- 27- CMVGFP/5 and in figure 5-27 for 1764 4- 27- pR19GFP.

Ideally, a positive control of infected cells treated with TSA or sodium butyrate would have been included in these assays, however, due to the limitations of size of the 96-well plate used for the qPCR, this was not possible.



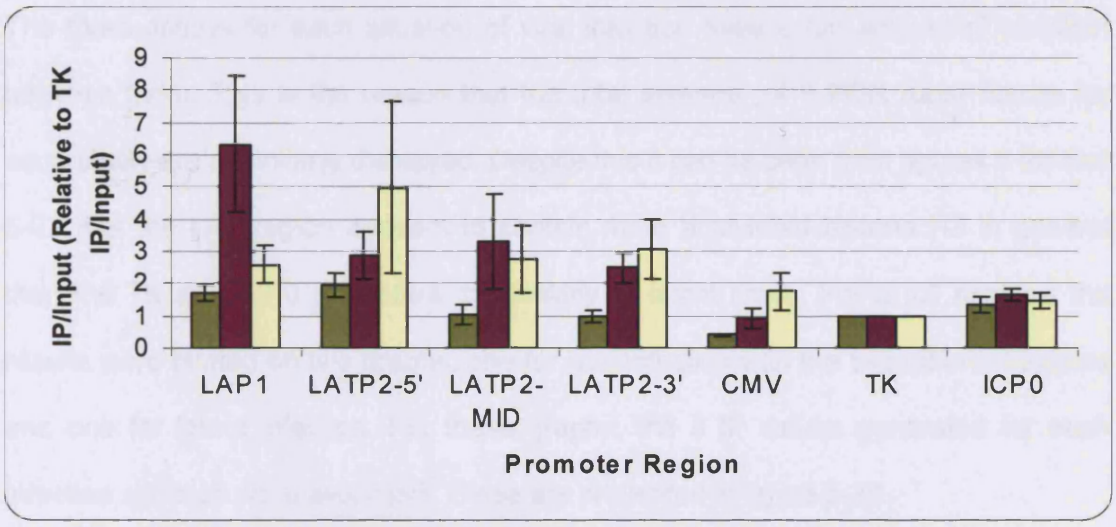
a. Lytic infection (3 hrs p.l.) – 27/12/M:4 cells



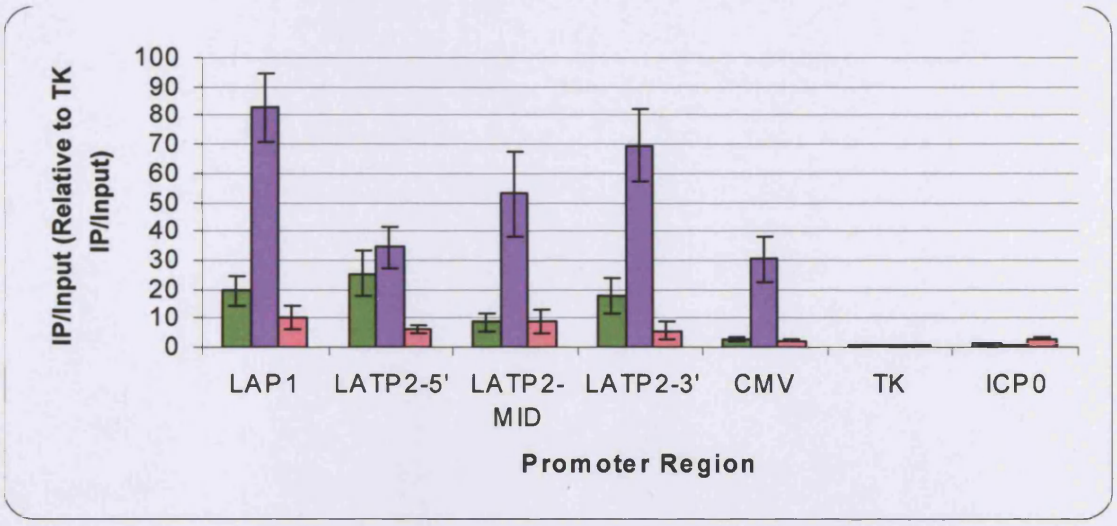
b. Latent infection (3 days p.l.) – ND7 cells

Figure 5-26 Acetylation values of promoters within 1764 4- 27- CMVGFP/5 during acute and latent infection

For each assay, 3 PCR runs were carried out and the means are displayed for each assay. Error bars represent the S.E.M.



a. Lytic infection (3 hrs p.I.)- 27/12/M:4 cells

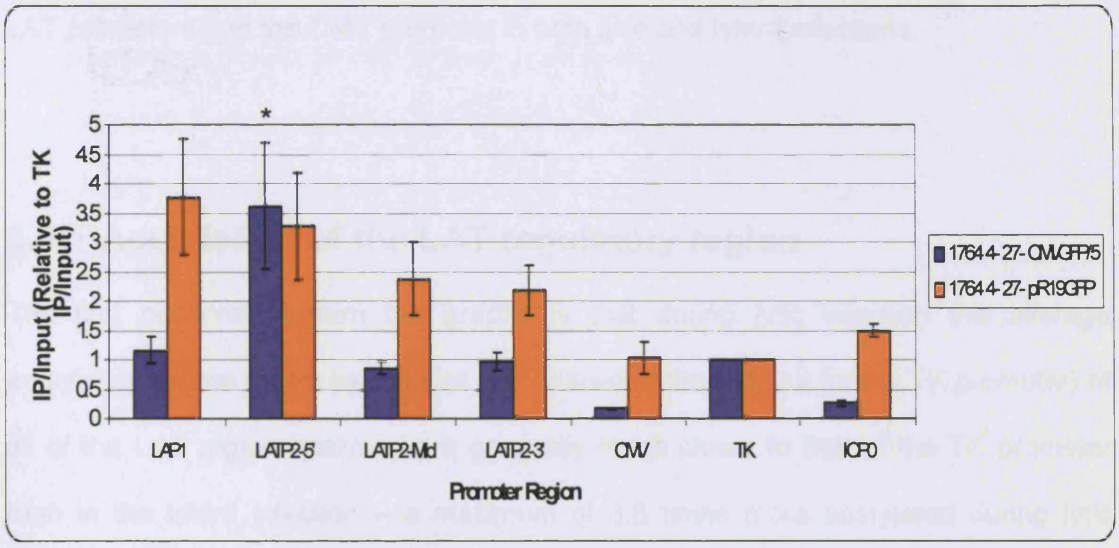


b. Latent infection (3 days p.I.) - ND7 cells

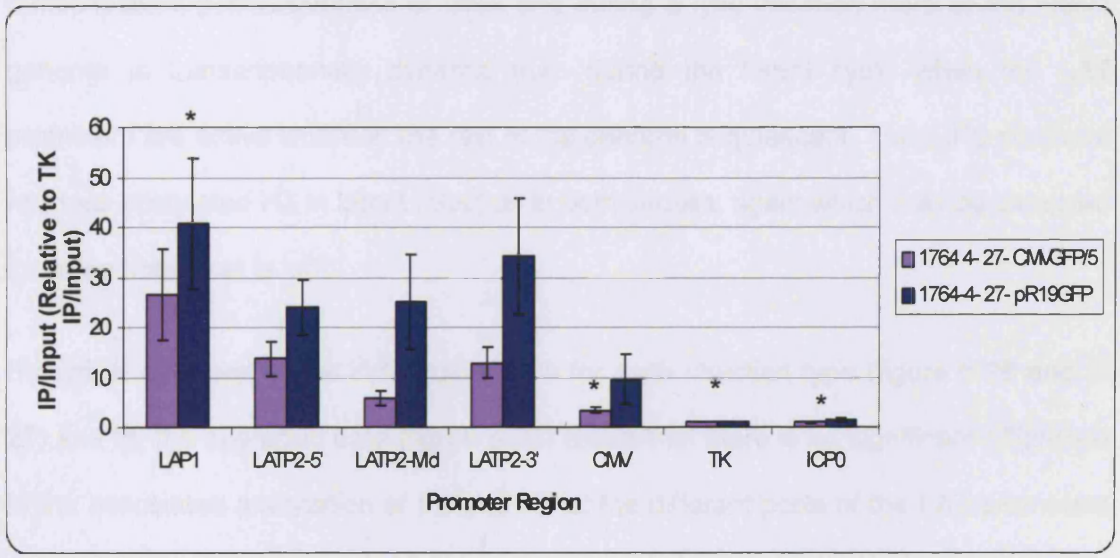
Figure 5-27 Acetylation values of promoters within 1764 4- 27- pR19GFP during acute and latent infection.

For each assay, 3 PCR runs were carried out and the means are displayed for each assay. Error bars represent the S.E.M.

The three assays for each situation of viral infection have a fair amount of variation between them. This is the reason that the total average (of 9 PCR runs) results for each assay are not initially displayed. Despite this it can be seen from figures 5-26 and 5-27 that the LAT region appears to contain more acetylated histone H3 in general than the TK and ICP0 promoters, particularly at latent times. For a full analysis the results were plotted on two graphs, one for lytic infection with the two different viruses and one for latent infection. For these graphs, the 9 IP values generated for each infection situation were averaged. These are presented in figure 5-28.



a. Lytic Infection (3 hrs p.i.)



b. Latent infection (3 days p.i.)

Figure 5-28 Comparison of acetylation of histone H3 at promoters in two different viral vectors

a. Lytic infection data
b. Latent infection data

Each data series represents the average of 3 assays per condition, with 3 qPCR runs each (i.e. n=9). Values are represented as relative to the IP/Input value for the TK promoter. (* - $p<0.05$)

Figure 5-28 shows the differences in acetylation of histone H3 at HSV1 LAT and non-LAT promoters and the CMV promoter in both lytic and latent infections.

5.9.1 Acetylation of the LAT regulatory region

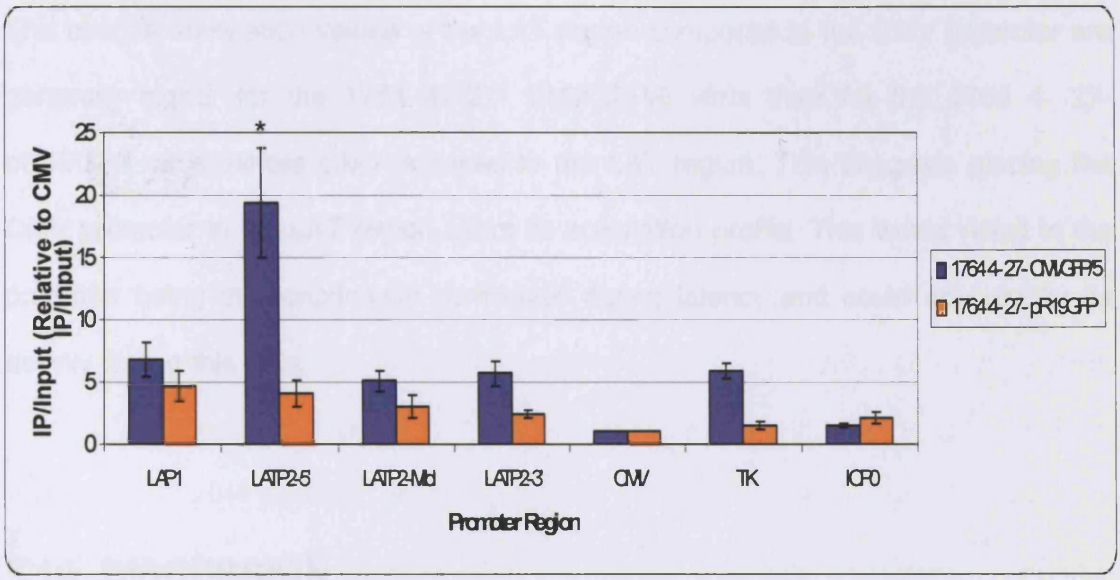
The first observation from the graphs is that during lytic infection the average acetylation values (given by IP/Input and relative to the IP/Input for the TK promoter) of all of the LAT region analysed are generally much closer to that of the TK promoter than in the latent infection – a maximum of 3.8 times more acetylated during lytic infection compared to 40.9 in latent infection (note the difference in scale between figure 5-28 a & b). This is perhaps unsurprising as acetylation is a marker for transcriptional permissiveness of DNA and during a lytic infection more of the HSV1 genome is transcriptionally dynamic than during the latent cycle when the LAT promoters are active whereas the rest of the genome is quiescent. The ICP0 promoter has less acetylated H3 in latent infection in both viruses, again which may be expected for a promoter that is 'off'.

Statistical analyses of the individual ChIPs for each infection type (figure 5-26 and 5-27) and for the averaged data (figure 5-28) reveal that there is no significant difference in the associated acetylation of histone H3 at the different parts of the LAT promoters – either LAP1 or any of the LATP2 region except for during lytic infection with 1764 4-27- CMVGFP/5 where the 5' half of LATP2 has a significantly ($p<0.05$) higher acetylation ratio than any of the other promoter regions studied. However this is not seen in lytic infection with the 1764 4- 27-pR19GFP virus and they would perhaps be expected to have a similar acetylation profile in this region. Nevertheless, the positioning of the two cassettes could have an effect.

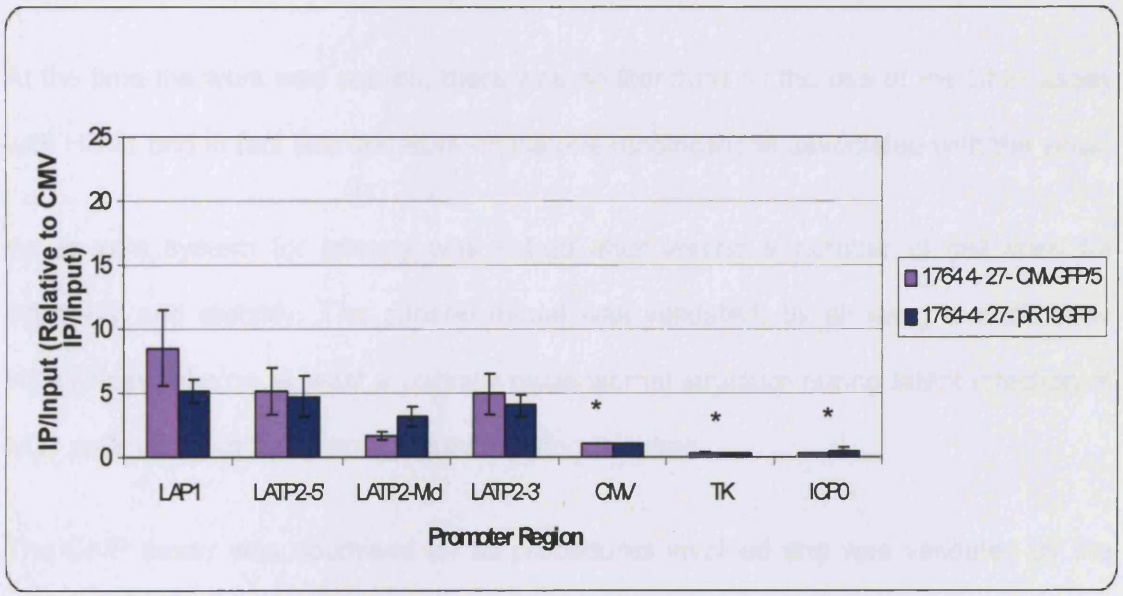
Comparing the two viruses in figure 5-28 shows that the LAT promoters studied in the 1764 4- 27- pR19GFP virus generally appear to be associated with more acetylated histone H3 than the TK promoter, in both lytic and latent situations, than in the 1764 4- 27- CMVGFP/5 virus. The fact that the 5' half of LATP2 in the 1764 4- 27- CMVGFP/5 does not fit this pattern perhaps means that this is an anomalous result. This suggests that in inserting the cassette containing CMV and GFP into the LAT region, the virus has been altered such that the region is more transcriptionally permissive. This is true for the ICP0 promoter only in a lytic infection, which is interesting as at that time the promoter is active, whereas during a latent infection it is inactive. This suggests that ICP0 is somehow 'protected' from the transcriptionally permissive neighbouring LAT region during latency, perhaps by an insulator element (see section 1.2.3.5). This data might suggest that this insulator is not present during lytic infection, allowing the ICP0 region to be influenced by changes in the local genome such as appears to have occurred in the 1764 4- 27- pR19GFP virus.

5.9.2 Acetylation of the exogenous CMV promoter

In order to assess whether the acetylation status of H3 at the CMV promoter is altered by placing it within the LAT region, compared to placing it outside of the LAT region, the difference in acetylation between it and the LAT region must be looked at in both cases. If the IP/Input values for the viral promoters are compared to that of the CMV promoter rather than the TK promoter this is easier to see (figure 5-29):



a. Lytic infection (3 hrs p.i.)



b. Latent infection (3 days p.i.)

Figure 5-29 Mean acetylation values (IP/Input) relative to CMV acetylation values in two different viral vectors

Each data series represents the average of 3 assays per condition, with 3 qPCR runs each (i.e. n=9). IP/input values are represented as relative to the CMV promoter IP/input values. (* - $p<0.05$).

The relative acetylation values of the LAT region compared to the CMV promoter are generally higher for the 1764 4- 27- CMVGFP/5 virus than for the 1764 4- 27- pR19GFP virus, where CMV is linked to the LAT region. This suggests placing the CMV promoter in the LAT region alters its acetylation profile. This would result in the promoter being transcriptionally permissive during latency and could account for its activity during this time.

5.10 DISCUSSION

This chapter described the optimisation and application of the ChIP assay for study of the acetylation of the HSV1 genome.

At the time the work was started, there was no literature on the use of the ChIP assay with HSV1 and in fact little literature on histone modifications associated with the virus.

An *in vitro* system for latency was set-up after testing a number of cell lines for infectivity and stability. The chosen model was validated, by showing that disabled HSV1 does take on at least a partially nucleosomal structure during latent infection of ND7 cells and that LATs are produced during this time.

The ChIP assay was optimised for all procedures involved and was validated by the use of PCR primers for the cellular gene cyclophilin. The use of standard PCR turned out to be limiting in detecting the acetylation of histone H3 associated with promoters in the latent infection due to the smaller amount of virus present. Therefore the use of quantitative PCR was looked at and proved to be a successful way of analysing the acetylated nucleosomes, of both latent and lytic *in vitro* infections.

The optimised ChIP assay was then used to analyse the acetylation profiles of two different viruses – 1764 4- 27-CMVGFP and 1764 4- 27-pR19GFP in which the CMV promoter was either in a cassette distinct from or linked to LATP2 respectively. The acetylation of histone H3 at the LAT regulatory region was also examined by using primers to the LAP1 promoter and to the different sections of the LATP2 region as studied in chapters 3 and 4. Acetylation at the ICP0 promoter was also looked at as it is both an IE gene and in close proximity to the LAT region. Acetylation at the TK promoter was studied, as it is an early gene promoter located away from the LAT region in the HSV1 genome.

The acetylation profiles of the two viruses were examined during acute infection and during latent infection. The LAT region promoters studied were enriched in acetylated histone H3 compared with the non-LAT promoters, particularly during latency. The rest of the genome is transcriptionally silent during this time and therefore this data shows that acetylation of histone H3 occurs where the viral genome is transcriptionally active. There were no consistent differences seen between the acetylation at the LAP1 promoter and at the sections of LATP2. This shows that this entire region is transcriptionally permissive during both lytic and latent infection. Acetylation is not a measure of promoter/enhancer activity, but rather the potential for activity, thus the two cannot be reconciled.

During the course of these experiments, a number of publications appeared in which acetylation of the HSV1 genome was examined in a similar manner. Kubat *et al.* showed that latent HSV1 DNA from mice DRG was associated with acetylated histone H3 at the LAP1 promoter whereas the HSV1 DNA polymerase, ICP4 and UL54 genes were not enriched in this acetylated histone (Kubat *et al.* 2004b). This concurs with the work described above in so much as the LAP1 promoter is enriched in acetylated histone H3 during latency compared to non-LAT regions. This paper also points out the fact, as mentioned above, that small changes in the fold enrichment of acetyl

histone H3 can result in huge changes in transcriptional activity of a promoter, as exemplified by a study in which quantification of hormone-induced hyperacetylation of H3 on transcription from the steroidogenic acute regulatory protein promoter was carried out (Christenson *et al.* 2001). This showed that a 5-fold increase in acetylation resulted in a 45-fold increase in mRNA accumulation and a 6-fold increase in acetylation resulted in a 125-fold increase in mRNA accumulation. This again would perhaps explain why differences in promoter/enhancer activity from the LATP2 region were seen in chapters 3 and 4, but no big differences in acetylation between the different parts are seen from the ChIP assays carried out above.

A second publication by Kubat *et al.* went on to study histone H3 acetylation at the LAT 5'exon by ChIP assay (Kubat *et al.* 2004a). This corresponds to part of the LATP2 region studied above, specifically, primers used for qPCR were localised to the section of LATP2 present in both the 5' half and mid-section. They showed that the 5' exon is at least as acetylated, if not more so, than the LAP1 promoter during latency, again by carrying out ChIP assays on HSV1 DNA from infected mice DRGs. They also showed that ICP0 is not as enriched in acetylated histone H3 as the LAT region at this time. The data also showed a similar amount of variation as in the experiments described in this chapter. These results again concur with the results from the *in vitro* latent infection above. This supports that the latent infection obtained in ND7 cells is a good model of a latent infection *in vivo* in terms of transcriptional activity and genome structure.

The work in this chapter also suggested that when the CMV promoter is placed adjacent to the LATP2 region, it becomes enriched in acetylated histone H3 compared to when it is placed in a non-LAT locus, during lytic and latent infection. A similar virus in which CMV was in the same position next to LATP2 but drove *lacZ* expression rather than GFP, allowed transgene expression during latency (Palmer *et al.* 2000). The CMV promoter does not allow this when placed elsewhere in the HSV1 genome.

This would suggest then that CMV becomes associated with modified histones in a similar manner to the LAT region and is allowed to remain active. The positioning of CMV next to LATP2 appears to allow this to occur. It was previously postulated that the altered dinucleotide content of the LAT region could reflect an altered DNA structure and that this could keep the chromatin in an 'open' transcriptionally permissive state (Coffin *et al.* 1995). Specifically, LATP2 could cause this alteration in structure and confer this onto neighbouring promoters. It now seems possible that this altered structure is to do with histone tail modifications in the region, specifically acetylation allowing the region to remain in an open, transcriptionally permissive state that is conferred onto exogenous promoters when placed next to LATP2.

Two other papers were published during the course of this work describing acetylation of H3 associated with HSV1. These looked at lytic infections, *in vitro.* The first group (Herrera and Triezenberg 2004) studied acetylation at 2 hours p.i. and found that the IE early promoters for ICP0, ICP4 and ICP27 were hypoacetylated on histone H3 at this time, but that promoters for delayed-early or late genes were hyperacetylated. When they repeated the study with a virus lacking the VP16 activation domain, they found that none of the viral promoters were acetylated at this time, suggesting that VP16 plays an important role in the epi-genetic modification of the genome during lytic infection.

The second study published at the same time (Kent *et al.* 2004), studied acetylated histone H3 at 1, 2 and 3 and 6 hours p.i. associated with IE, E, and L gene promoters and showed a correlation between timing of RNA appearance for the genes and acetylation of the promoters. This provides further evidence for the role of histone modification in the regulation of HSV1 transcriptional patterns.

The results presented in this chapter shows that LAP1 is apparently as enriched in acetylated histone H3 as LATP2 during latency, yet does not confer latent expression

onto exogenous promoters as LATP2 does. It would be interesting to see whether LAP1 would be as enriched in acetylated histone H3 if it were placed at a different locus in the genome than next to LATP2, i.e. whether or not LATP2 is responsible for this acetylation status. The fact that LAP1 is turned off during latency when LATP2 is not adjacent suggests that it would not. LATP2 contains enhancer activity, and this may well be due to or causing more acetylation of H3 in the associated nucleosome(s) at this locus. The finding in chapter 3 that there is a binding site for the TF p300 that contains HAT activity within the 3' half of LATP2, gives weight to this hypothesis.

The fact that the CMV promoter appears to become enriched in acetylated histone H3 when placed next to LATP2 does suggest that this histone modification is involved in conferring latent activity. It seems that the MMLV LTR plays a similar role to LATP2 in maintaining long-term activity from the LAP1 promoter and so it would be interesting to see whether the MMLV LTR also has/causes an increase in the acetylation of associated histones.

The fact that LATP2 can confer activity onto neighbouring promoters leads to the point that although the ICP0 promoter is proximal to the LAT region, it is not enriched in histone H3 during latency and thus LATP2 is not conferring latent expression onto this neighbouring promoter. It is an attractive hypothesis therefore, that there is a factor present such as an insulator element between LATP2 and ICP0, which would prevent this open chromatin structure from being spread to the ICP0 gene, or more importantly the closed heterochromatin structure of the rest of the genome spreading to the LAT region. Insulator elements are also commonly found in the proximity of enhancers for the purpose of preventing enhancer activity on promoters where it is not wanted. If there is such an insulator element present, the CMV promoter in the 1764 4-27-pR19GFP virus must have been placed upstream of it, in order to allow activity during latency.

In conclusion, the LAT regulatory region including LATP2 does appear to be enriched in acetylated histone H3 as compared to the non-LAT promoters studied, particularly during latency and furthermore this enrichment appears to be conferred onto the CMV promoter when placed next to the LATP2 promoter/enhancer.

CHAPTER 6:

DISCUSSION

HSV-1 is a good candidate for a gene therapy vector, particularly for delivering genes to the nervous system. The beneficial characteristics making it an appealing vector to develop include the ability to have large gene sequences inserted, to naturally infect neurons and to sustain a latent infection for the lifetime of the host.

The harnessing of the virus' ability to maintain transcriptional activity from a small genetic region asymptomatically is a key aim in the development of vectors with the ability for continued transgene expression. Various groups have achieved this with greater or lesser success, by utilising the LAT regulatory region or parts thereof to drive continued gene expression.

The LATP2 region has previously been shown to be responsible at least partly for maintaining long-term expression of inserted transgenes and has been used in our laboratory to achieve continued expression in the nervous system from two different promoters placed in a bicistronic arrangement to this region.

The work presented in this thesis attempted to characterise the LATP2 region in order to understand better its mechanism of action such that future vectors may be designed with optimised long-term expression capabilities.

The first two results chapters (chapters 3 and 4) presented here investigated which regions of LATP2 are responsible for its enhancer and long-term expression characteristics. The third results chapter (chapter 5) examined whether the virus uses epigenetic mechanisms to maintain transcriptional activity of the LAT region during latency.

In chapter 3, vectors were created that contained LATP2 deletion mutants in a central position within a cassette, flanked by the LAP1 promoter and the MMLV LTR promoter on either side driving *lacZ* and GFP respectively. Through *in vitro* and *in vivo* testing, repressive elements were suggested in the 5' half and 3' half of LATP2 not

encompassing the mid fragment i.e. the extreme ends. This appeared to be functioning during lytic and latent infection. However, the fact that expression from both promoters was at its highest without LATP2 at all suggested that the MMLV enhancer activity was conferring long-term expression onto LAP1 (a feature of the MMLV LTR that had previously been reported) and being repressed by the presence of LATP2. Thus the enhancer activity of LATP2 was not conclusive, as it was potentially being obscured by the presence of the MMLV LTR.

Therefore, in chapter 4, the MMLV LTR and GFP were removed from the LATP2 deletion viruses used in chapter 3, giving a set of viruses with the LATP2 deletions in the opposite orientation to LAP1, driving *lacZ* expression. A further series of LATP2 deletion viruses were also created with the same deletions, but orientated in the same direction as LAP1, to allow study of the directionality of the region. When these vectors were tested *in vitro* and *in vivo*, the data again suggested a repressive element, although this time in the mid section of LATP2, which only caused repression when in the same orientation as LAP1. It is not clear exactly where this was located as neither the 3' half or the 5' half alone seemed to exhibit this repression. It is possible that in whichever half it is, it contains active elements that balance the effects out. However, removing the 5' half of LATP2 seemed to cause an increase in enhancer activity, therefore the repressive region is perhaps more likely to be located in the region of LATP2 common to the 5' half and mid-section.

As mentioned, the mid section of LATP2 tested repressed LAP1 activity when in the same orientation as LAP1. However, it actually enhanced the activity when placed in the opposite orientation. This seems unusual, but it appears that a sequence has been deleted from LATP2 that would allow the region to function in both directions. This appears to have created a unidirectional enhancer, which although unusual, has been reported in the literature before (Carlberg *et al.* 1988; Gerasimova *et al.* 1995; Hen *et al.* 1983). It is also possible that the joining of this deletion fragment to the adjacent

DNA, in the same orientation as LAP1 has allowed a site for an insulator element or for a repressor to bind, thus preventing the enhancement of LAP1 activity in one orientation only. Some directionality is also seen from the full LATP2 region and the 5'LATP2ΔICP4 fragment, suggesting that maybe the region does not possess equally bi-directional activity and that deletion of certain parts emphasises this.

As stated above, the 5' half of LATP2 generally exhibited no enhancer activity, whilst the 3' half was required for enhancer activity in most situations tested and long-term expression. The mid section was able to enhance LAP1 activity when in the opposite orientation to LAP1 *in vivo*, therefore it seems that only the first 242bp of LATP2 exhibits no enhancer activity. This is in contrast to similar work carried out by Berthomme *et al* who showed that the 5' half of this region was sufficient for full enhancer activity of the region whilst the 3' half exhibited none (Berthomme *et al.* 2001). This could be explained by the different arrangement of the LAT promoter and enhancer elements in the two studies. It is possible that introducing deletions either destroys or creates binding sites for repressive or active elements, as does joining different sections of DNA, which could have occurred in either or both sets of experiments resulting in different enhancer profiles. Further to this it was reported that an enhancer element could be shared between LAP1 and LAP2 (Soares *et al.* 1996), which if this were so could explain why enhancer activity was not seen from the 5' half of LATP2 in the work in this thesis as the arrangement of LAP1 and LAP2 here would have disrupted this element.

A question posed by Berthomme *et al* was whether the enhancer and long-term expression functions are the same or distinct. Enhancers often function by creating an open conformation of the local chromatin structure. If this is so in LATP2 then it seems difficult to imagine that the long-term expression function is different from the enhancer function, i.e. the long-term expression that it allows is probably due to continued opening of the region as directed by the enhancer. The work presented here suggests

that they are the same. The fragments that enhance activity from LAP1 at lytic timepoints also tend to allow enhanced long-term expression. The other group investigating this region arrived at the same conclusion from their experiments, even though the different arrangement pointed to different active or repressive regions (Berthomme *et al.* 2001).

The full LATP2 region gives the best long-term expression of a transgene from LAP1 when in the same orientation as LAP1. Taken together with the results from the deletion fragments, this suggests that the LATP2 region is made up of a complex linear array of *cis*-acting elements, more than one of which is required for optimal transgene expression during latency.

Having said this, future work should include creation of further LATP2 deletion mutants with both the section unique to the 5' half deleted (i.e. the first 242bp) and separately, the section common to the 5' and mid-section (~400bp) deleted and introduced into similar vectors to examine whether the repressive region can be further localised and deleted to produce optimal long-term expression vectors for delivery to the PNS.

In chapter 5, the hypothesis that the LAT regulatory elements, specifically LATP2, maintain the local region in a transcriptionally active state by means of histone modifications was tested. It is known that HSV1 is associated with histones during latency (Deshmane and Fraser 1989) and acetylated histones are a marker of transcriptional activity; therefore acetylation of the LAT promoter and enhancer were compared to that of non-LAT promoters. The acetylation status of a non-HSV promoter that has previously been shown to remain active when placed next to LATP2 was examined, to also see whether this is achieved by the acetylation of associated histones.

In vitro models of lytic and latent infection were set-up and it was shown that HSV1 is at least partially associated with nucleosomes during both of these infections. The ChIP assay on HSV-1 infected cells was optimised and then used to examine the acetylation of histone H3 associated with two different viruses, containing a CMV promoter either within the LAT region or placed elsewhere in the genome.

The assays showed that the LAT promoter and enhancer regions are more associated with acetylated histone H3 than non-LAT promoters during latency. The different parts of LATP2 do not appear to have differently acetylated histones to each other, but this is maybe not surprising when a number of factors are considered. As mentioned previously, changes in promoter/enhancer activity do not necessarily have a linear relationship with changes in the acetylation of associated histones. Therefore, small changes in the activity of different sections of LATP2 will probably not be detectable in the acetylation of the associated histones. As well as this, the fact that acetylation of histones is a marker of the transcriptional ability of the associated DNA rather than the actual transcriptional activity shows that the region as a whole is active. Furthermore, as the data from chapter 4 suggests, it appears that elements throughout LATP2 function to keep the region active. Activating or repressing element motifs usually consist of a maximum of about 15-20 bp whereas each nucleosome has 146bp of DNA wound round it, therefore each two H3 histones contained in a nucleosome could have many differently acting elements associated with them.

In chapter 3, a putative binding site for the TF p300 was identified in the DNA unique to the 3' half of LATP2. p300 has HAT activity and could therefore be involved in the acetylation of this region. This is backed up by the fact that in chapter 4, the 3' half of the region was required for long-term activity and was able to allow continued expression without the 5' half of LATP2. This would correspond to the presence or absence of this p300 binding site which could allow p300 to confer acetylation onto the associated histones and lead to continued activity.

The CMV promoter showed a selective association with hyperacetylated histone H3 during latency when linked to the LATP2 promoter, as compared to when it was placed in the U_S5 gene. Therefore, it appears that LATP2 may confer long-term expression onto the CMV promoter by allowing it to stay in a transcriptionally active open conformation, via acetylation of associated histone H3 at a time when it would otherwise be shut off.

This continued acetylation might be achieved by inhibition of HDACs during latency. How the virus might bring this about remains to be discovered. Presumably some mechanism perhaps modulated by the LATs, which are the only viral product at this time, functions to prevent this deacetylation and switching off of the region.

It seems quite likely that the distance that continued activity can be conferred onto proximal promoters by LATP2 is limited by a barrier element. The fact that the ICP0 promoter is shut off during latency and is hypoacetylated at histone H3 suggests that a barrier may exist somewhere between LATP2 and ICP0 to prevent this action of LATP2 on the IE gene promoter. In fact, very recent evidence has shown that this is likely, as a CTCF insulator protein motif cluster has been identified exactly in this location; between LATP2 and ICP0 (Amelio and Bloom 2005). It would be interesting to delete these motifs and see the effect on activity of ICP0 during latency and indeed on the establishment of latency.

Similarly, in the region upstream of LAP1, there must be another barrier to prevent chromatin condensation from the rest of the genome spreading to the LAT region from this direction, as barriers exist in pairs to create distinct domains of hetero- or eu-chromatin.

An interesting experiment to conduct would be to insert an artificial barrier between LATP2 and LAP1 or CMV and examine the effect on transgene expression driven by

these promoters during latency. This would allow confirmation that it is LATP2 that the acetylated, transcriptionally permissible chromatin originates from.

As seen in chapter 3 and in previous reports, the MMLV LTR also has the ability to confer activity onto LAP1, as does LATP2, making it an attractive hypothesis that it functions in a similar manner to LATP2. It would be interesting to examine the acetylation status of histones associated with MMLV LTR when linked to LAP1 in a non-LAT locus (an arrangement that allows long-term expression) to discover whether this is so.

As LATP2 can be placed outside of the LAT region and confer long-term expression onto related promoters it would also be interesting to examine the acetylation of histones in surrounding regions (e.g. in U_L43 as in the chapter 3 and 4 viruses) to see how far this extends from LATP2, as barriers presumably exist in the proximity of the inserted regions to prevent shut-off of promoters within the cassettes during latency. Either the cassettes have benefited from fortuitous placement, or LATP2 somehow prevents shut-off by another means. It is unlikely that this is due to fortuitous placement, as the latent transgene expression can be conferred by LAT elements whether in the U_L43 locus (Palmer *et al.* 2000), the U_S5 locus, or gC (Berthomme *et al.* 2001) in a similar manner.

However this compartmentalisation is achieved, placement of transgene expression cassettes within HSV1 vectors would potentially benefit from the introduction of barrier protein binding elements at each end to ensure that inappropriate genes are not kept on during latency. This should perhaps be considered in future HSV1 vector design.

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